TREATMENT AND CHEMOPREVENTION OF FAMILIAL DUODENAL ADENOMATOSIS

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ISBN: 978-94-6182-325-0

Layout and printing: Off Page, www.offpage.nl

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This research project was financially supported by the Dutch Cancer Society (KWF Kankerbestrijding), grant KUN 2008-4198.

The publication of this thesis was financially supported by: AbbVie B.V., Cobra Medical B.V., Dr. Falk Pharma, Ferring Pharmaceuticals, Nederlandse Vereniging voor Gastroenterologie, Olympus Nederland B.V., Radboud University Nijmegen Medical Centre, Roche Diagnostics Nederland B.V., Tramedico B.V., Vifor Pharma Nederland B.V., and Zambon Nederland B.V.

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Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. mr. S.C.J.J. Kortmann,
volgens besluit van het college van decanen
in het openbaar te verdedigen op
woensdag 23 oktober 2013
om 10.30 uur precies

door

Bjorn Wilhelmus Harry van Heumen
geboren op 4 november 1976
te Nijmegen

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CHAPTER 1

General introduction

Abbreviations: AFAP, attenuated familial adenomatous polyposis; *APC*, adenomatous polyposis coli; CA, cholic acid; CDCA, chenodeoxycholic acid; CHRPE, congenital hypertrophy of the retinal pigment epithelium; COX, cyclooxygenase; DFMO, difluoromethylornithine; EPA, eicosapentaenoic acid; FAP, familial adenomatous polyposis; IPAA, ileal pouch-anal anastomosis; IRA, ileorectal anastomosis; Lef, lymfhoid enhancer factor; MAP, MYH associated polyposis; MCR, mutation cluster region; NSAID, non-steroidal anti-inflammatory drug; PDT, photodynamic therapy; PPPD, pylorus-preserving pancreaticoduodenectomy; PSD, pancreas-sparing duodenectomy; Tcf, T cell factor family; TGF-α, transforming growth facter α; UDCA, ursodeoxycholic acid; Whipple, Whipple's pancreaticoduodenectomy

FAMILIAL ADENOMATOUS POLYPOSIS

Familial adenomatous polyposis (FAP) is an inheritable disease that is characterized classically by the development of hundreds to thousands adenomatous polyps in the colorectum during the second and third decade of life.[1] In approximately 10% of cases, the disease is less severe, with a lower number of colorectal polyps and a higher average age of onset of disease. This variant is called attenuated FAP (AFAP).[2] Virtually all patients with FAP will develop colorectal cancer before the age of 40 to 50 years, unless prophylactic colectomy is performed. At present, ileorectal anastomosis (IRA) and ileal pouch-anal anastomosis (IPAA) are the surgical procedures of choice.

In addition to colorectal abnormalities, patients with FAP are at risk for extra-colonic manifestations of their disease. These include epidermoid cysts, lipomas, desmoid tumors, osteomas, dental abnormalities, congenital hypertrophy of the retinal epithelium, adrenal tumors, thyroid carcinomas, brain tumors, pancreatic cancer, hepatoblastomas and upper gastrointestinal adenomas and carcinomas.[3,4] By convention, the clinical association of FAP with desmoid tumors and osteomas is referred to as Gardner syndrome, whereas Turcot syndrome is characterized by the association of FAP with tumors of the central nervous system, in particular medulla blastoma.

All clinicians involved in management of patients with FAP should be aware of all these possible extra-intestinal manifestations, as FAP related complications for which medical attention is essential, are not rare and their estimated lifetime risk is presumed to exceed 30%.[4]

Based on data from Northern European Polyposis registries, FAP has a reported prevalence of 26 to 32 patients per million, an incidence of 0.86 to 2.38 patients per million per year, and a frequency at birth of approximately one in 7,000 to one in 25,000.[5-7] In the past decades, prognosis of FAP has improved considerably, owing to a substantial reduction in the prevalence of colorectal cancer, as a result of the establishment of numerous national and regional registers all over the world.[8,9] The centralized registration has facilitated the identification of family members at risk and early diagnosis. Prophylactic colectomy substantially improved prognosis in the past decades.[10] As a result, the mortality pattern has changed with duodenal cancer and desmoid tumors now being the main cancer-related causes of death.[11-14] In this thesis, studies are described that address treatment and chemoprevention of duodenal neoplasms, primarily in patients with FAP.

UPPER GASTROINTESTINAL MANIFESTATIONS OF FAP

Duodenal polyps were first described by Funkenstein in the year 1904[15] and the first case of a FAP related duodenal carcinoma was reported by Cabot in 1935.[16] Although interest for upper gastrointestinal polyposis grew after the first publication in the late 1960s, suggesting regular upper gastrointestinal surveillance for patients with FAP to detect polyps, even after the introduction of endoscopy in the 1970s surveillance was not performed systematically.[17] The first publication of upper gastrointestinal endoscopic screening was published in 1977.[18]

Gastric lesions associated with FAP frequently are fundic gland polyps, while gastric adenomas are found in 6% of patients.[19] An increased risk of gastric cancer is not found in patients with FAP compared to the general population.[20] Data on the prevalence of duodenal adenomas in patients with FAP vary widely with rates from 20 to 100%, depending on the type of endoscope used and the method of tissue sampling.[17,21,22] With the use of side-viewing endoscopy and random biopsies of endoscopically normal appearing mucosa, remarkably high rates of duodenal and periampullary adenomas of over 70% were found, with a considerable prevalence of microadenomas in papillary and periampullary mucosa.[21] Polyps can be found throughout the duodenum, but the second and third segment and the periampullary region are most commonly affected. This distribution corresponds with mucosal exposure to bile acids, suggesting that these substances are involved in duodenal carcinogenesis.[23,24]

Duodenal adenomatosis is generally graded according to the endoscopically and histologically based semi-quantitative scoring system by Spigelman.[23] In this scoring system, severity of duodenal polyposis is expressed according to the endoscopic evaluation of number and size of the polyps, in combination with histology and grade of dysplasia in biopsies of duodenal lesion. The scoring system describes five (0-IV) stages (see ***Table 1***). Stage I (1-4 points) indicates mild disease, whereas stages III-IV (>6 points) imply severe duodenal polyposis.

Table 1. Spigelman classification for duodenal adenomatosis with recommendations for management.[22,25]

	Points		
Criterion	**1**	**2**	**3**
Number	1-4	5-20	>20
Size (mm)	1-4	5-10	>10
Architecture	Tubular	Tubulovillous	Villous
Dysplasia	Mild	Moderate	Severe

Stage 0 (0 points) and stage I (1-4 points): endoscopic surveillance every 5 years; stage II (5-6 points): endoscopic surveillance every 2-3 years; stage III (7-8 points): endoscopic surveillance every 1-2 years with consideration for surgery; stage IV (9-12 points): surgery should be considered.

The Spigelman classification of duodenal adenomatosis allows duodenal polyposis to be compared over time and between observers, and is helpful in estimating the cancer risk. Progression of duodenal adenomatosis is slow, particularly in patients with less advanced stages of disease.[25-28] In two large studies, no malignant progression was present after 10 years of follow-up in patients with Spigelman stage 0 or I.[25,27] In these studies, the cumulative incidence of malignancy was 0.7-1.9% for patients with Spigelman stages 0 to III, and 7-36% for patients with Spigelman stage IV. The risk of developing stage III or IV disease exponentially increases after the age of 40.[28] In patients reaching 75 years of age, 20% to 52% had developed Spigelman IV duodenal adenomatosis, with an estimated cumulative incidence of duodenal

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adenocarcinoma between 4 and 33%.[25,29] This illustrates that, in contrast to colorectal polyps, duodenal polyps do not invariably progress to adenocarcinomas. However, the relative risk for duodenal adenocarcinoma or ampullary carcinoma in patients with FAP was estimated 331 and 124 times higher respectively, as compared to the general population.[20] In general, it is assumed that life-time risk of duodenal carcinoma in patients with FAP is 2-7%.[21,25,27,29,30] Noteworthy, an increase in severity of duodenal polyposis as reported in recent years is determined by time-lapse, technological advances, and changes in dysplasia-reporting. These changes suggest that a revision of the Spigelman classification is warranted.[31]

APC GENETICS AND FUNCTION

FAP is an autosomal dominant disease caused by germline mutations in the tumor suppressor *adenomatous polyposis coli* (*APC*) gene.[32] Approximately 25% of all cases of FAP are caused by *de novo* mutations, which means there is no family history of colorectal lesions or identified *APC* germline mutation.[33] Linkage analysis of families with FAP led to the localizing of the *APC* gene on chromosome 5.[32] In 1991, the gene was identified on chromosome 5q21-22 and further characterized.[34-36] The *APC* gene consists of 8,538 base pairs spanning 15 exons, encoding a protein consisting of 2,843 amino acids. Exon 15 is the largest exon containing over 75% of the coding sequence.[35]

With conventional techniques for genetic testing, in approximately 20-50% of patients with clinical presentation of FAP or AFAP no mutation can be detected.[37] More recently, a mutation in the *MYH* gene, involved in oxidation-induced DNA damage repair, was identified in *APC* mutation-negative patients with multiple colorectal polyps.[38] This *MYH* associated polyposis (MAP) with an autosomal recessive pattern of inheritance could account for up to 30% of *APC* mutation-negative polyposis patients.[39] Other germline mutations may contribute to the *APC* mutation-negative group as well, e.g. mutations in the *CRAC1* or *AXIN2* genes.[40,41] Moreover, the severity and progression of adenomatosis may not be determined by the *APC* gene alone. The discovery of a modifier *Mom1* gene in the *APC*^{Min} mouse polyposis model[42] led to the identification of a possible modifier gene on human chromosome 1p35-36, which may contribute to the clinical heterogeneity of FAP.[43,44] Variations in the *N*-acetyltransferase loci *NAT1* and *NAT2*, located on chromosome 8p22, have also been shown to affect disease severity.[45]

In accordance with Knudson's 'two-hit' hypothesis[46], patients with FAP inherit one germline mutation and develop tumors from cells in which a 'second hit' or loss of the other *APC* allele (loss of heterozygosity) is acquired somatically.[47] Somatic mutations in the colorectal polyps of patients with FAP do not seem to occur randomly. Over 60% of all somatic mutations in *APC* occur clustered in the region between codons 1286 and 1513, therefore called the colorectal 'mutation cluster region' (MCR).[48] The localization of the germline mutation also seems to determine the type of somatic 'second hit' mutation in colorectal polyps: germline mutations close to codon 1300 are associated with loss of heterozygosity, and germline mutations elsewhere in the gene are associated with truncating somatic mutations in the MCR.[47] The vast majority of germline mutations in the *APC* gene result in a truncated nonfunctional protein, either by a nonsense mutation (30%) or by a frameshift mutation (68%), with most of the

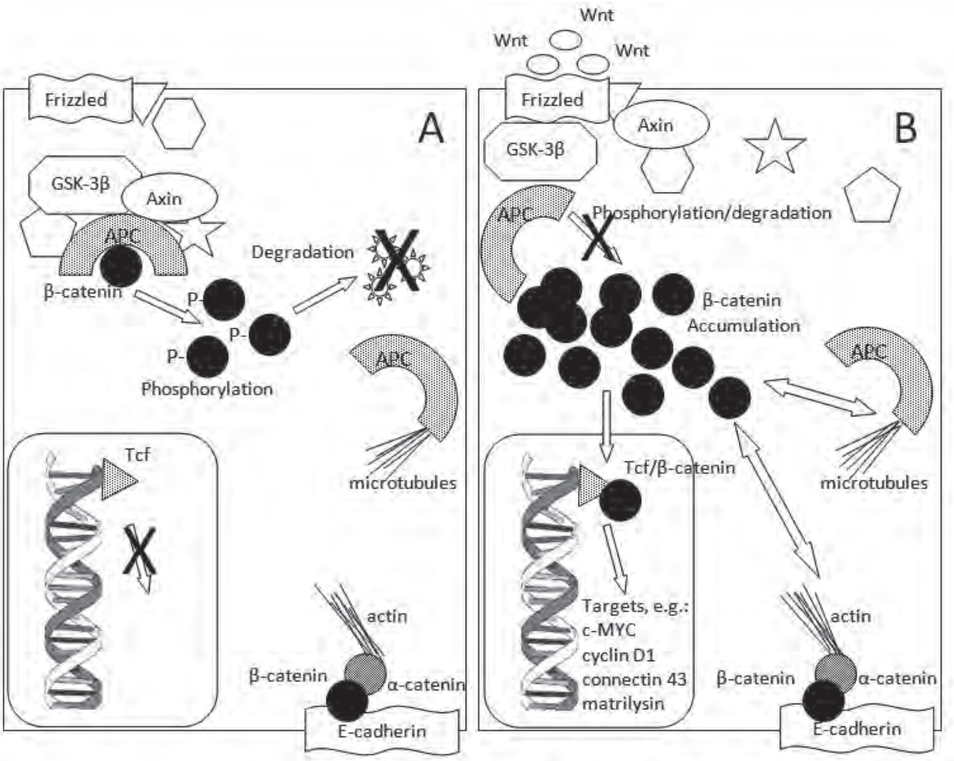

Figure 1. APC function and the Wnt signaling/β-catenin pathway - adapted from Fearnhead *et al.*[52] A: In the absence of Wnt ligand and with normal functioning APC, β-catenin is phosphorylated by GSK-3β in a complex with APC and Axin. Phosphorylated β-catenin is rapidly degradated. Furthermore, β-catenin is associated with E-cadherin and α-catenin which binds actin and actin-associated proteins of the microtubule cytoskeleton. B: Inactivated GSK-3β by binding of Wnt ligand to its receptor (known as Frizzled) or loss of functional APC, results in accumulation of cytosolic β-catenin, which translocates to the nucleus. Inside the nucleus, β-catenin associates with members of the T cell factor (Tcf) and lymphoid enhancer factor (Lef) family of transcription factors. The Tcf/β-catenin complex activates several transcriptional targets, including c-MYC, cyclin D, connectin 43, and matrilysin. In addition, other cellular functions of APC and β-catenin are also disturbed.

mutations (germline and somatic) occurring in the first half of the coding region of the gene.[49] Germline mutational hotspots are located at codons 1061 and 1309.[49,50]

The *APC* gene encodes a large protein with multiple domains related to multiple cellular processes. It is involved in oligomerization, cell migration and adhesion, β-catenin binding and degradation, axin binding, microtubule binding, chromosome segregation, cell cycle regulation and apoptosis.[51,52] It is hypothesized that the critical tumor suppression activity involves the intracellular Wnt signaling pathway, more specifically by downregulation of β-catenin through phosphorylation and degradation in association with axin and GSK-3β (see ***Figure 1***).[53,54] Loss of functional *APC* results in accumulation of cytosolic β-catenin and subsequent translocation to the nucleus.[52] Inside the nucleus, β-catenin associates with members of the T cell factor

1 2 3 4 5 6 7 8 9 &

(Tcf) and lymphoid enhancer factor (Lef) family of transcription factors, e.g. Tcf4 which is expressed in the nuclei of intestinal epithelial cells. The β-catenin/Tcf4 complex activates several transcriptional targets, including the oncogene and cell cycle regulator *c-MYC*[55], the G1/S-regulating gene *cyclin D1*[56], the gap junction protein connectin 43[57], and the gene encoding the matrix-degrading metalloproteinase matrilysin.[58] In addition to controlling the Wnt signaling pathway, APC has other functions. In this regard, β-catenin also functions as an essential component of epithelial intercellular adherens junctions, where it links the cytoplasmic tail of E-cadherin to α-catenin which binds actin and actin-associated proteins of the microtubule cytoskeleton.[59] In summary, the role of APC in intestinal carcinogenesis is attributed largely to the Wnt signaling pathway, but disruption of intercellular adhesion and stability of the cytoskeleton seems to be involved as well.[52]

ADENOMA-CARCINOMA SEQUENCE

'The adenoma-carcinoma sequence' is the term for the histological stepwise progression of normal colorectal mucosa to aberrant crypt foci, adenoma, and finally invasive colorectal cancer (see ***Figure 2***). The stepwise progression of normal colorectal mucosa to carcinoma in patients with FAP is paralleled by a series of genetic and cellular changes that involves activation of oncogenes (e.g. *K-ras*) and inactivation of tumor suppressor genes (e.g. *p53* and *SMAD4*).[59] Based on epidemiological data of differences in age between presentation with benign and malignant neoplasms, a similar adenoma-carcinoma sequence was suggested to apply also to carcinogenesis in the small intestine including the duodenum.[60] Histopathological evidence for this includes the finding in patients with FAP, that adenomas occurred either as a component of duodenal carcinomas or in mucosa adjacent to duodenal carcinomas in 84% of patients, suggesting that a carcinoma is preceded by a noninvasive adenomatous precursor lesion.[30]

The biallelic mutations in the *APC* gene with activation of the Wnt signaling pathway are the earliest genetic alterations and seem to be required to initiate the sequence, both in hereditary predisposed patients with FAP, as well as in sporadic carcinomas.[62] Findings that support the concept of progressive cellular alteration include the following. First, no somatic 'second hit'

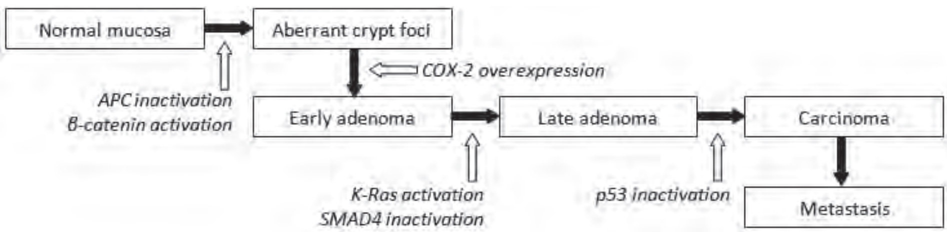

Figure 2. Colorectal adenoma-carcinoma sequence - adapted from Brosens *et al.*[61] The histological stepwise progression of normal colorectal mucosa to aberrant crypt foci, adenoma, and finally invasive colorectal cancer, is paralleled by a series of genetic and cellular changes that involve activation of oncogenes (e.g. K-ras) and inactivation of tumor suppressor genes (e.g. p53 and SMAD4). A similar adenoma-carcinoma sequence is suggested to apply to carcinogenesis in the duodenum.

APC mutations were detected in normal duodenal mucosa of patients with FAP[63], but were observed in 10-67% of periampullary adenomas and carcinomas.[63-65] Second, oncogenic *K-ras* mutations were detected in duodenal adenomas and carcinomas.[64-67] Third, overexpression of p53 was noted in 0% of normal mucosa, 25% of tubular, 72% of tubulovillous or villous adenomas, and 100% of duodenal carcinomas.[68] As detection of *p53* gene mutation is generally low or absent, p53 overexpression rather seems to reflect intracellular abnormalities possibly related to tumorigenesis.[66,67] Fourth, several cellular abnormalities were found to be already present in normal duodenal mucosa of patients with FAP when compared to non-FAP controls, including increased cell proliferation[69,70], increased expression of cyclooxygenase-2 (COX-2)[71], increased number of Paneth cells and endocrine cells in the mucosal crypts[72], and loss of extracellular E-cadherin.[73] Fifth, the extent of transforming growth factor α (TGF-α) expression was found to be greater in duodenal carcinomas than in adenomas, and increased progressively in adenomas relative to the degree of dysplasia (mild, moderate, severe) and histological architecture (tubulous, tubulovillous, villous) of these lesions.[74] Altogether, these studies, at least to some extent, reveal the molecular and genetic alterations that are involved in the transition of normal duodenal mucosa into adenoma and carcinoma.

GENOTYPE-PHENOTYPE CORRELATION

The correlation between the location of germline mutations in the *APC* gene and the clinical presentation in patients with FAP, the genotype-phenotype correlation, was first recognized and described in relation to the number of colorectal polyps in 1992.[75] The phenotype with profuse colorectal polyposis of thousands (over 5000) polyps was linked to mutations between codons 1250 and 1464. In the attenuated phenotype AFAP with less than one hundred polyps, mutations were reported at the 5' end spanning exons 4 and 5 before codon 157, within the alternative spliced region of exon 9, and at the 3' end of the gene beyond codon 1595.[76] An intermediate phenotype of one hundred to several thousand polyps was correlated to the rest of the gene (see ***Figure 3***). In addition to the severity of colorectal polyposis, the age of onset was also found to be associated with the localization of the APC germline mutation. Patients carrying an *APC* mutation at codon 1309, a germline mutational hotspot as mentioned before, showed an age of onset of disease 10 years earlier (mean age of 20 years) compared with patients with other mutations between codons 168 and 1580 (mean age of 30 years), whereas patients with mutations at the 5' end of codon 168 or the 3' end of codon 1580 were diagnosed at a mean age of 52 years.[50]

Genotype-phenotype correlations of extra-colonic manifestations have not been well established, with the exception of the occurrence of congenital hypertrophy of the retinal pigment epithelium (CHRPE), which is restricted to families with mutations between codons 311 and 1465.[76] The multiple bilateral presence of these ophthalmic lesions appears to be a highly specific marker for FAP and can therefore be used to identify carriers in FAP families.[77]

In relation to upper gastrointestinal polyps, evidence for a genotype-phenotype correlation is not conclusive. In several studies, no association was found between localization of *APC* mutations and duodenal polyposis.[50,78,79] However, there is evidence suggesting that proximal mutations of the *APC* gene are related to a smaller risk of duodenal adenomas compared to mutations downstream, that is beyond codon 479[80], or on codons 564-1465.[81] A three to fourfold increased risk of duodenal adenomas for patient with *APC* gene mutations on codons 976-1067 was found as compared to patients with mutations on codons 159-495.[82] Moreover, highest frequencies of gastric and duodenal adenomas and periampullary cancer was associated with mutations on codons 1395-1493.[83] A history of periampullary adenomas or adenocarcinomas was associated with mutations downstream of codon 1051.[84]

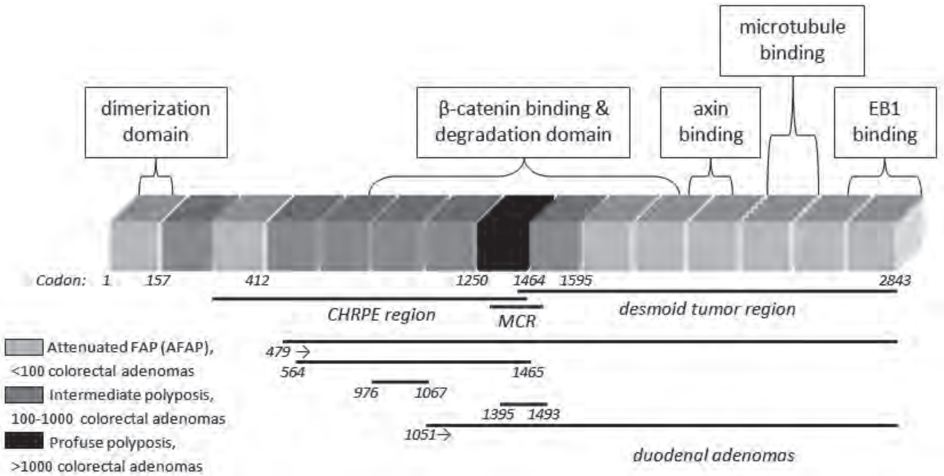

Figure 3. Genotype-phenotype correlation: the localization of mutations in the APC gene in association with the clinical colorectal phenotype - adapted from Nieuwenhuis *et al.*[85] Evidence on genotype-phenotype of duodenal adenomas however is not straightforward.[80-84] *Abbreviations*: CHRPE, congenital hypertrophy of the retinal pigment epithelium; MCR, mutation cluster region.

MANAGEMENT OF DUODENAL ADENOMATOSIS

The clinical challenge is to identify patients with high-risk duodenal adenomas and intervene before progression to cancer has occured. Therefore, surveillance gastroduodenoscopy is recommended from around the age of 25-30 years, ideally using a side-viewing video-endoscope for optimal view of the ampullary region, and including taking biopsies of mucosal lesions as well as randomly.[22] Surveillance interval and treatment according to the Spigelman stage is depicted in ***Table 1***.

Endoscopic treatment options for duodenal lesions include snare excision, thermal ablation, argon plasma coagulation, and photodynamic therapy (PDT). In contrast to colorectal polyps, duodenal adenomas are often flat non-polypoid structures and therefore difficult to remove using conventional snare excision.[61] Submucosal injection of saline/adrenaline before removal is a strategy that reduces risk of haemorrhage and perforation.[27] Local treatment of duodenal polyposis with polypectomy or ampullectomy, endoscopically as well as surgically, is a relatively safe option, but high adenoma recurrence rates of up to almost 100% have been reported.[86-89] The relief of cancer threat therefore seems only temporary and ongoing endoscopic surveillance is mandatory. Moreover, patients that were previously treated for Spigelman stage IV demonstrated an increased disease progression, as compared to patients with natural disease progression, and for these former stage IV patients, more frequent surveillance is advised than general recommendations based on current Spigelman stage.[90] When local treatment is no longer deemed possible, more extensive surgical procedures such as classical Whipple's pancreaticoduodenectomy (Whipple), pylorus-preserving pancreaticoduodenectomy (PPPD) or the more recently introduced pancreas-sparing duodenectomy (PSD) need to be considered.[91-93] However, these interventions bring about substantial risk of morbidity and mortality.[92,94-96] Furthermore, although these extensive surgical procedures offer the chance of a prolonged disease-free interval, recurrence of adenomas[97,98] and even cancer arising from the remaining duodenal mucosa have been reported.[99] Moreover, cancer recurrence at the hepaticojejunostomy was described thirteen years after PPPD for nonampullary duodenal cancer, as described in **Chapter 2** of this thesis.[100] Chemopreventive treatment is highly desirable to postpone or even avoid the necessity for radical prophylactic surgery, or as adjuvant treatment after prophylactic duodenal resection is performed.

CHEMOPREVENTION

Theoretically, all cellular processes involved in initiation or progression of adenomas or carcinomas are potential targets for pharmacological intervention in the carcinogenesis. Cyclooxygenase (COX) inhibiting non-steroidal anti-inflammatory drugs (NSAIDs) have been subject of much investigation as potential chemopreventive agents. Two COX isoenzymes exist. Whereas the COX-1 isoenzyme is constitutively expressed in a wide range of tissues and is considered a housekeeping enzyme, the COX-2 isoenzyme is an inducible enzyme that produces prostaglandins in inflammatory and tumorigenic settings.[101] Overexpression of COX-2 is linked to evasion of apoptosis, enhanced cell growth, tumor angiogenesis, tissue invasion, and metastasis through several signalling pathways.[101]

In 1983, the first report was published on regression of colorectal polyps in a patient with FAP treated with sulindac, a non-selective cyclooxygenase inhibitor.[102] Subsequently, in the first randomized controlled trial, sulindac was found to significantly reduce the number of rectal polyps, as compared to placebo.[103] The effects of sulindac were also examined in the duodenum, but results were disappointing. Of the 8 patients with FAP with large duodenal polyps treated with sulindac for 6 months, 3 patients discontinued treatment because of significant side effects, in 3 patients large polyps recurred, and in one patient even invasive cancer developed.[104] However, sulindac was found to regress small duodenal polyps, but this effect was limited, despite larger effects on colorectal polyposis.[105,106]

The inhibition of the COX-1 isoenzyme appears to cause the major side effects common to NSAIDs, in particular the gastrointestinal complications.[107] Subsequently, studies in which COX-2 was specifically targeted by administration of the selective COX-2 inhibitor celecoxib, showed significant decrease in the occurrence of sporadic colorectal adenomas, not only by suppressing the growth of existing adenomas, but also by preventing the formation of new adenomas.[108] COX-2 inhibition in murine models of intestinal polyposis resulted in a substantial decrease in adenoma size and number.[109,110] Confirming the findings from animal studies, administration of celecoxib was associated with regression of adenomas of both the colon and rectum in patients with FAP.[111]

The value of COX inhibiting agents for regression of duodenal polyposis however, is not well established.[61] Celecoxib was found to significantly reduce duodenal adenomatosis in a subset of patients with FAP with significant duodenal adenomatosis, after 6 months of treatment with high dosage of 400mg twice daily[112], a finding that was confirmed by us, as described in **Chapter 6** of this thesis. Unfortunately, due to increased risks of adverse cardiovascular events, clinical trials involving selective COX-2 inhibitors as chemopreventive agents for colorectal cancer have cast doubt on the suitability of these agents for long-term use.[108,113]

The search for effective chemopreventive treatment is ongoing and strategies of investigational interest in patients with FAP include sulindac and difluoromethylornithine (DFMO)[114], curcumin and quercetin[115], and eicosapentaenoic acid (EPA).[116] Another promising strategy is combining celecoxib, preferably in lower dosage regimens to minimize cardiovascular toxicity, with other substances such as ursodeoxycholic acid (UDCA).

As previously mentioned, distribution of duodenal polyps corresponds with mucosal exposure to bile acids, suggesting their involvement in carcinogenesis.[23,24] Primary bile acids are synthesized in the liver, excreted in the biliary tract, and released into the duodenum for digestive processes. In the terminal ileum, most of the bile acids are reabsorbed. Only approximately 5% of the bile acid pool enters the colon, where intestinal flora is responsible for the transformation of primary bile acids to secondary bile acids. These secondary bile acids are known tumor promotors in the gastrointestinal tract.[117,118] After prophylactic colectomy in patients with FAP is performed, the content of the circulating bile acid pool changes and gallbladder and duodenal bile largely constitutes of glycine or taurine conjugated cholic acid (CA) and chenodeoxycholic acid (CDCA) derivates, both in equivalent amounts.[119,120] By treating patients with FAP with high dosage of UDCA, enrichment with UDCA of duodenal bile of up to 50% of the total amount of bile acids was reached, with a reduction in concentration of the cytotoxic bile acid CDCA.[121] In *in vitro* models of human colonic epithelial cells, UDCA, and taurine-conjugated UDCA in particular, significantly reduced cytotoxicity of secondary bile acids.[122] Data from clinical studies support the notion of a possible chemopreventive effect of UDCA on development of colorectal neoplasms, in patients with sporadic colorectal adenomas[123] and patients with ulcerative colitis and primary sclerosing cholangitis.[124,125] UDCA was found to reduce COX-2 expression in a rat model of colon carcinogenesis[126], suggesting an alternative and possibly complimentary pathway for inhibition of COX-2.[127] However, in a recent clinical trial in patients with FAP, no effect of low dose UDCA after 24 months as mono-treatment was found on Spigelman scores.[128] Interestingly, a synergistic effect of sulindac and UDCA in the prevention of intestinal adenomas was found in a murine model of FAP.[110] In this thesis, the chemopreventive potential of the combination of celecoxib and UDCA is studied *in vitro* as well as *in vivo*, as described in **Chapter 4**, and **Chapters 6** & **7**, respectively.

OUTLINE OF THIS THESIS

The main objectives of this thesis are:

1. To evaluate the management and its outcome of sporadic duodenal adenomas and duodenal adenomatosis in patients with FAP, as employed in past decades, to further define their clinical significance and implication, and its management.
2. To explore the chemopreventive effects on duodenal adenomatosis of potentially effective substances in a preclinical setting, either as single treatment or in combination, for development of future chemopreventive strategies in patients with FAP.
3. To investigate the chemopreventive effects on duodenal adenomatosis in patients with FAP of treatment with celecoxib and UDCA in a multicentre randomized clinical trial.

Management and follow-up of duodenal adenomatosis, as it was practiced in the past decades, is studied in the first section of this thesis. In **Chapter 2**, surgical management and follow-up of advanced duodenal adenomatosis and duodenal cancer in patients with FAP is reviewed retrospectively, using data from the Dutch polyposis registry in Leiden. In **Chapter 3**, results are presented of the retrospective review of management and follow-up of sporadic duodenal adenomas, as it was employed at our institute over the past decades.

In the second section, potential chemopreventive strategies for management of duodenal adenomatosis in patients with FAP are explored by *in vitro* studies on gastrointestinal tumor cell line models. In **Chapter 4**, the chemopreventive potential of curcumin, quercetin and EPA are investigated by studying their effects on expression and activity of the important detoxification enzymes glutathione S-transferase and UDP-glucuronosyltransferase in cells of four different gastrointestinal tumor cell lines. In **Chapter 5**, celecoxib and UDCA co-treatment is studied by evaluating effects on cell growth and COX-2 mRNA expression in two different colorectal tumor cell lines.

In the third section of this thesis, results of a multicentre randomized clinical trial are presented. The effect of celecoxib and UDCA co-treatment on duodenal adenomatosis in patients with FAP is evaluated in a randomized, double-blind study in patients with Spigelman stages II or III duodenal adenomatosis, receiving either celecoxib & UDCA or celecoxib & placebo treatment. **Chapter 6** focuses on the endoscopic and histopathological effects of the two treatment arms in this intervention study. In **Chapter 7**, effects on duodenal mucosal mRNA levels of nine potential risk parameters for malignant transformation are studied in the two treatment arms in patients with FAP. In addition, baseline levels in duodenal biopsy specimens of patients with FAP are compared with levels in non-FAP patient controls.

In **Chapter 8**, the results described in this thesis are summarized and reviewed, and future perspectives are postulated. **Chapter 9** presents a summary of this thesis in Dutch.

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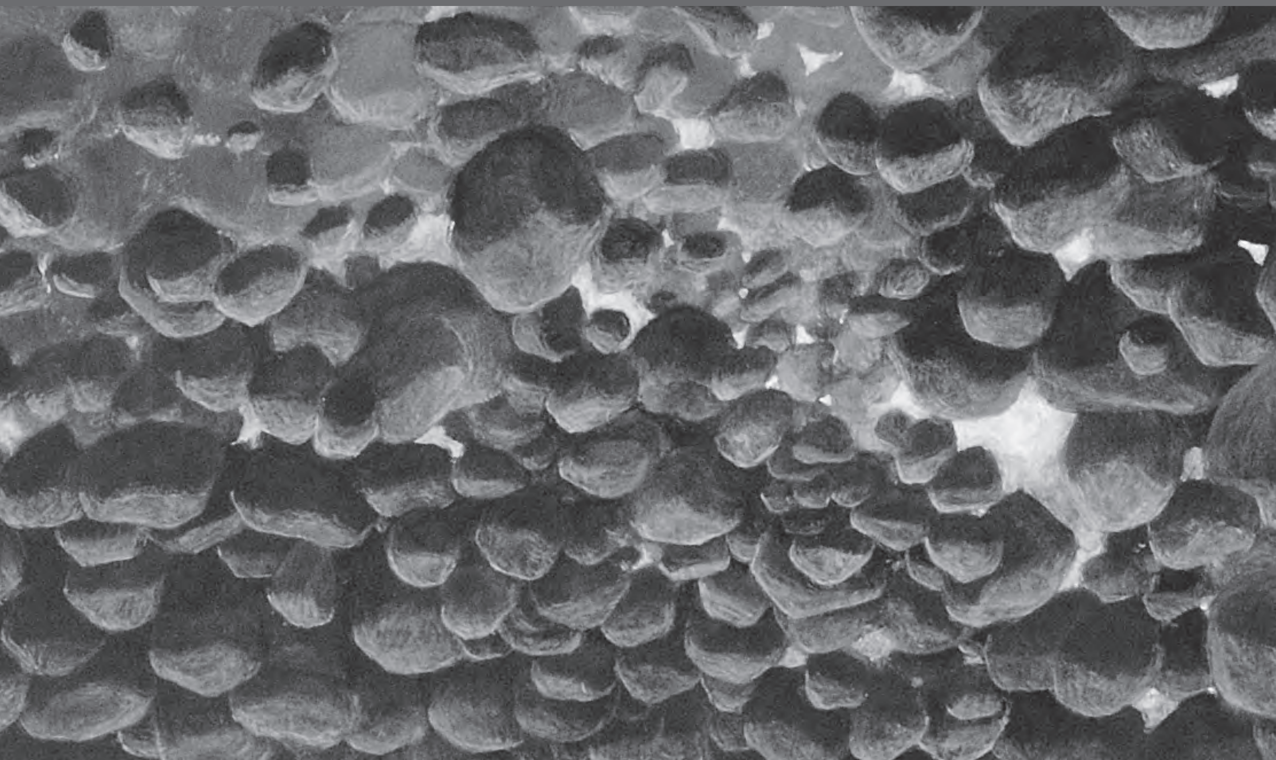
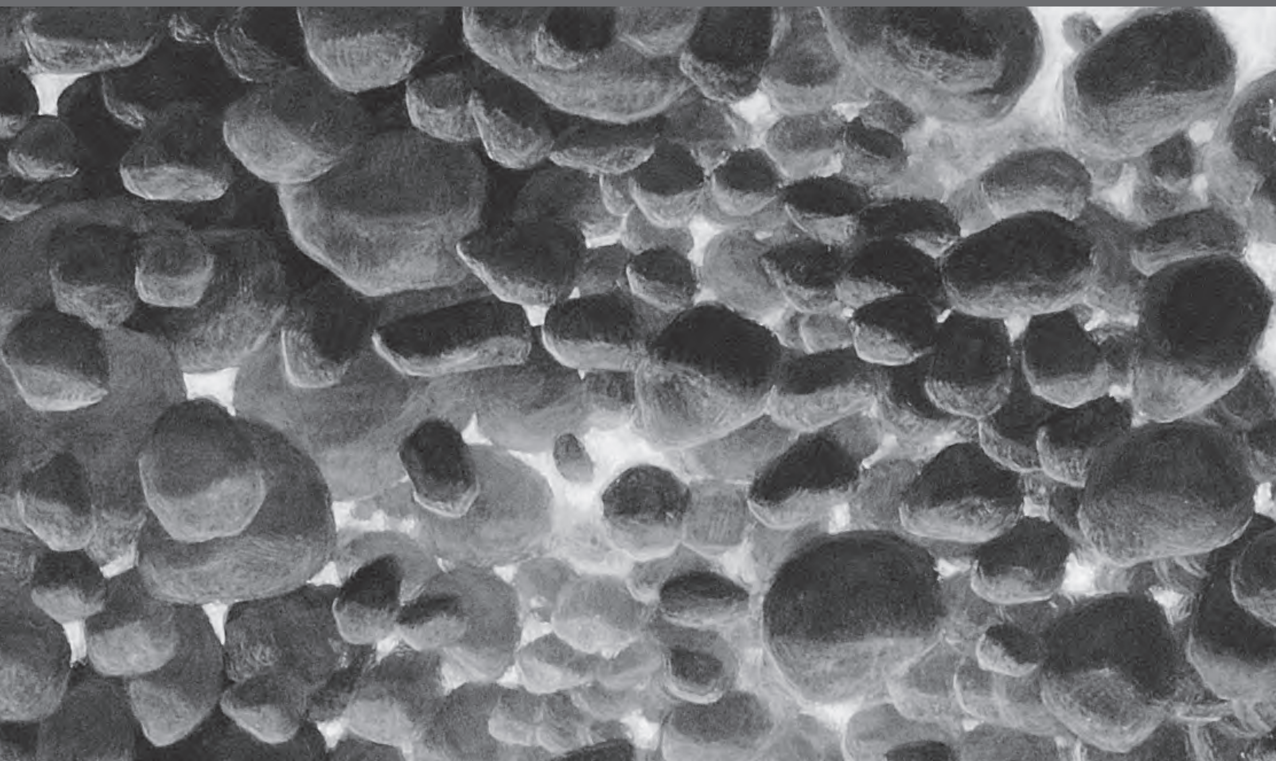


SECTION A

Adenomas in the duodenum: retrospective analysis of management



CHAPTER 2

Surgical management for advanced duodenal adenomatosis and duodenal cancer in Dutch patients with familial adenomatous polyposis: A nationwide retrospective cohort study

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PUBLISHED IN: *SURGERY*, 2012, 151(5): 681-90.

ABSTRACT

Duodenal cancer is a major cause of mortality in patients with familial adenomatous polyposis (FAP). The clinical challenge is to perform duodenectomy before cancer develops. However, procedures are associated with complications. Our aim was to gain insight into the pros and cons of prophylactic duodenectomy. Patients with FAP from the nationwide Dutch polyposis registry who underwent prophylactic duodenectomy or were diagnosed with duodenal cancer were identified and classified as having benign disease or cancer at preoperative endoscopy. Surveillance, clinical presentation, surgical management, outcome, survival, and recurrence were compared. Of 1,066 patients with FAP in the registry, 52 (5%; 25 males) were included: 36 with benign adenomatosis (median: 48 years old; including two (6%) cancer cases diagnosed after operation), and 16 with cancer (median: 53 years old). Cancer cases had been diagnosed with colorectal cancer more often (6% vs 44%; p<.01). Forty-three patients underwent duodenectomy (35 benign/eight cancer): 30-day mortality was 4.7% (n=2), and in-hospital morbidity occurred in 21 patients (49%), without differences between patients with benign adenomatosis and cancer. Adenomas recurred in reconstructed proximal small bowel in 14 of 28 patients (50%, median time to recurrence: 75 months), and one patient developed cancer. Median survival of all 18 cancer cases in the registry (1.7%; 12 ampullary/six duodenal) was 11 months. In conclusion, prognosis of duodenal cancer in patients with FAP is poor, which justifies an aggressive approach to advanced benign adenomatosis. Strict adherence to recommended surveillance intervals is essential for a well-timed intervention. Given the substantial morbidity and mortality of duodenectomy, patients' individual characteristics are to be critically evaluated preoperatively. As adenomas recur, postoperative endoscopic surveillance is mandatory.

Keywords: familial adenomatous polyposis; duodenal adenomatosis; prophylactic duodenectomy; duodenal cancer; surveillance; surgical outcome; survival; recurrence

Abbreviations: APC, adenomatous polyposis coli; FAP, familial adenomatous polyposis; FDG-PET, fludeoxyglucose positron emission tomography; IPAA, ileal pouch-anal anastomosis; IRA, ileorectal anastomosis; NFDHT, Netherlands Foundation for Detection of Hereditary Tumours; PPPD, pylorus-preserving pancreaticoduodenectomy; PSD, pancreas-sparing duodenectomy; Whipple, Whipple's pancreaticoduodenectomy

Acknowledgements: The authors thank Ms MEJ Gerrits-Eijck and Ms ME Velthuizen for their ongoing efforts of managing the NFDHT database, and Ms AY Jung for reviewing the manuscript as native English speaker.

Familial adenomatous polyposis (FAP) is an autosomal-dominant disease caused by germline mutations in the tumor suppressor gene *APC* (*adenomatous polyposis coli*).[1] Classically, FAP is characterized by the development of hundreds to thousands of adenomatous polyps in the colorectum.[2] Unless a prophylactic colectomy is performed, virtually all patients will develop colorectal cancer before the age of 50 years. In the past, surveillance and prophylactic colorectal surgery for patients at risk improved prognosis substantially by preventing colorectal cancer.[3-6] As a result, duodenal cancer is now the main cancer-related cause of death in patients who underwent prophylactic colectomy.[7-9]

Although the lifetime risk of duodenal adenomas approaches 100% in patients with FAP,[10] in contrast to colorectal polyps, duodenal polyps do not inevitably transform to cancer. Approximately 3-4% of patients eventually develop duodenal cancer.[11,12] The relative risk of duodenal adenocarcinoma and ampullary carcinoma in patients with FAP was estimated at, respectively, 331 and 124 times greater than in the general population in which duodenal carcinoma is rare.[13]

The clinical challenge is to identify the patients at high risk of developing duodenal cancer because duodenal cancer has been associated with a poor prognosis.[11,14-16] The Spigelman stages of duodenal disease severity assessed by surveillance endoscopy have been shown to correlate with the risk of developing duodenal cancer, with a risk of 36% during a 10-year period for the most advanced stage IV.[17] Endoscopic surveillance is recommended to start when the patient is 25-30 years of age, and frequency of surveillance and further management are determined on the basis of the Spigelman classification (***Table 1***).[10,18]

Local treatment of duodenal polyposis with polypectomy or ampullectomy, endoscopically as well as surgically, is a relatively safe option, but high rates of adenoma recurrence up to almost 100% have been reported.[19-22] The relief of cancer threat, therefore, seems only temporary, and ongoing endoscopic surveillance is mandatory. When local treatment is no longer deemed possible, more extensive operative procedures such as classical pancreatoduodenectomy, pylorus-preserving pancreatoduodenectomy (PPPD), or the more recently introduced pancreas-sparing duodenectomy (PSD) need to be considered.[23] These interventions, however, bring about substantial risk of morbidity and mortality.[24-26] Furthermore, although these extensive surgical

Table 1. Spigelman classification for duodenal adenomatosis with recommendations for management.[10,18]

	Criterion points		
	1	**2**	**3**
Number	1-4	5-20	>20
Size (mm)	1-4	5-10	>10
Architecture	Tubular	Tubulovillous	Villous
Dysplasia	Mild	Moderate	Severe

Stage 0 (0 points) and stage I (1-4 points): endoscopic surveillance every 5 years; stage II (5-6 points): endoscopic surveillance every 2-3 years; stage III (7-8 points): endoscopic surveillance every 1-2 years with consideration for surgery; stage IV (9-12 points): surgery should be considered.

procedures offer the chance of a prolonged disease-free interval, the recurrence of adenomas[15,27] and even cancer arising from the remaining duodenal mucosa have been reported.[28]

To gain insight into the pros and cons of prophylactic duodenectomy, we reviewed data retrospectively of patients from the nationwide Dutch polyposis registry. Our primary aim was to analyze characteristics of all patients who underwent prophylactic duodenectomy for duodenal adenomatosis and all patients diagnosed with duodenal cancer. In addition, we compared both groups on features including surveillance and clinical presentation, surgical management and outcome, survival and causes of death, and recurrence.

PATIENTS AND METHODS

Dutch polyposis registry

In 1985, the Netherlands Foundation for Detection of Hereditary Tumours (NFDHT) started a registry of patients with FAP. The main objectives of this nationwide polyposis registry are coordination of lifelong surveillance of at-risk patients and promotion of early detection of cancer in high-risk families.[29] Patients with FAP are referred to this national registry by gastroenterologists, surgeons, or clinical geneticists. At the time of registration, written informed consent is obtained from the patient for collection of personal and medical data, including endoscopic, surgical, and histopathology reports. To date, 1,066 patients with a genetically and/or clinically confirmed diagnosis of FAP are registered.

Study population

For this study, we searched the FAP database of the Dutch Polyposis Registry to identify and include for analysis: (1) patients who underwent a classic pancreatoduodenectomy with antrectomy (Whipple), PPPD, or PSD for advanced benign duodenal adenomatosis; and (2) patients who presented with duodenal cancer, irrespective of whether duodenal surgery was performed. Patients were classified in 2 groups according to tumor status at preoperative endoscopy, as patients with benign duodenal adenomatosis or patients with duodenal cancer.

Definitions and description of variables

Available medical data were evaluated, including clinical correspondence and endoscopic, surgical, and histopathology reports. The following data were recorded: type and indication of previous colorectal resection, *APC* mutation status, and details on endoscopic assessments, in particular on the assessment before duodenal surgery and follow-up assessments after duodenal surgery. Cancers were classified as either ampullary or duodenal; duodenal cancers were those that arose from nonampullary duodenum. Moreover, the mode of presentation was reviewed, assessing whether cancers were detected at surveillance endoscopy or whether cancer patients presented with disease signs or symptoms. The recommendations as depicted in ***Table 1*** were generally used as standard for endoscopic surveillance in the Netherlands.

Complications after duodenectomy were classified as either in-hospital morbidity or long-term morbidity. Postoperative mortality was defined as 30-day mortality. Data on causes of death were collected, and causes were grouped as either related or unrelated to duodenal disease.

The recurrence of adenoma was defined as the appearance of new adenomas after duodenectomy (Whipple, PPPD, PSD) in the reconstructed proximal small bowel involving the residual duodenal mucosa (after PPPD or PSD) and the proximal jejunum used for reconstruction. Patients with adenomas at the first postoperative follow-up endoscopy were excluded, because these adenomas might have been present before duodenectomy was performed. Time to adenoma recurrence was measured from date of resection until date of first endoscopic surveillance showing the recurrence of adenomas or the date of last endoscopic surveillance without recurrence. Any carcinoma arising in the reconstructed proximal small bowel was also recorded.

Statistical analysis

Statistical analysis was performed using SPSS statistical software version 16.0 (SPSS, Chicago, IL). Frequency tables were provided for description of baseline characteristics. A group comparison was performed, primarily on the basis of tumor status at preoperative endoscopy. In addition, comparisons included groups defined by type of duodenectomy, surveillance status, and cancer localization. Differences on continuous variables including age at duodenal surgery were examined using Mann-Whitney U test. The Fisher's exact test was used to compare discrete variables, including those representing complications after duodenectomy, and causes of death. Survival and adenoma recurrence data were analyzed by Kaplan-Meier survival analysis and Log Rank test. A p-value of <.05 (2-sided) was considered statistically significant.

RESULTS

Description of the study population

Of the 1,066 patients with FAP in the registry database, 53 patients (5%; 26 male, 27 female) met the criteria for inclusion. One male patient with duodenal cancer was excluded from all analyses because of missing clinical data. Subsequently, the study population comprised 52 patients (25 male, 27 female) from 44 FAP families. The presence of a germline APC mutation had been confirmed by genetic testing in 44 patients (85%). Patient characteristics of the total study population and the subgroups classified by tumor status at preoperative endoscopy as either benign duodenal disease (n=36) or duodenal cancer (n=16), are summarized in ***Table 2***. Patients' tumor status, surgical approach, and outcome are shown in ***Figure 1***.

All operative procedures were performed in large regional teaching hospitals or specialized university centers between 1975 and 2008. The following procedures were performed: Whipple (n=13), PPPD (n=8), PSD (n=22), duodenotomy with ampullectomy (n=1), and laparotomy with or without palliative intervention because of unresectable and/or metastatic cancer (n=7). In one patient, an operation was not performed because of unresectable ampullary cancer. Nearly all PSDs were performed in the most recent decade (***Figure 2***). Operative therapy for benign duodenal adenomatosis was performed at a median age of 48 years and duodenal cancer surgery at a median of 53 years (Mann-Whitney U test, p=.23).

Compared with patients with benign disease at preoperative endoscopy, patients with duodenal cancer at endoscopy had been diagnosed with colorectal cancer at primary colorectal surgery more often (6% vs 44%, respectively; Fisher's exact test, p<.01).

Table 2. Characteristics of the study population consisting of patients with FAP with benign duodenal adenomatosis and duodenal cancer at endoscopy.

	Total study population	Cases with benign disease at endoscopy	Cases with duodenal cancer at endoscopy
Number of patients	52 (48% Male)	36 (47% Male)	16 (50% Male)
Median age at primary CR surgery (yr)	28 (range: 12-63)	28 (range: 12-49)	32 (range: 15-63)
Type of primary CR surgery			
IRA	31 (60%)	21 (58%)	10 (62.5%)
IPAA	12 (23%)	10 (28%)	2 (12.5%)
Ileostomy	9 (17%)	5 (14%)	4 (25%)
Cases with cancer at primary CR surgery	9[(1)] (17%)	2 (6%)	7[(1)] (44%)
Rectum	3	-	3
Colon	8	2	6
Median age at duodenal surgery (yr)	49 (range: 31-69)	48 (range: 31-69)	53 (range: 32-67)
Spigelman stage at preoperative endoscopy			
II	1	1	-
III	6	6	-
IV	29	29	-
Cancer	16	0	16
Duodenal cancer			
Ampullary	12	2	10
Duodenal	7	0	6
Type of duodenal surgery			
Whipple	13 (25%)	8 (22%)	5 (31%)
PPPD	8 (15%)	5 (14%)	3 (19%)
PSD	22 (42%)	22 (61%)	-
Duodenotomy with ampullectomy	1 (2%)	-	1 (6%)
Laparotomy with/without palliative intervention	7 (14%)	1 (3%)	6 (38%)
No surgery, irresectable cancer	1 (2%)	-	1 (6%)

[(1)] In 2 cases synchronous cancer of colon and rectum was present at primary colorectal surgery.
Abbreviations: CR, colorectal; IRA, Ileorectal anastomosis; IPAA, Ileal pouch-anal anastomosis; Ileostomy, Proctocolectomy with ileostomy; Whipple, classical Whipple's pancreaticoduodenectomy; PPPD, pylorus preserving pancreaticoduodenectomy; PSD, pancreas-sparing duodenectomy.

Patients with benign duodenal adenomatosis at preoperative endoscopy

The 36 patients with benign duodenal adenomatosis at preoperative endoscopy were graded as follows: Spigelman II disease with high-grade dysplasia around the neopapilla 9 years after ampullectomy (n=1), and Spigelman III disease (n=6) or Spigelman IV disease (n=29). All patients were under surveillance except for one who underwent endoscopy for dyspepsia and was found to have Spigelman IV disease. Three patients previously had undergone surgical treatment for

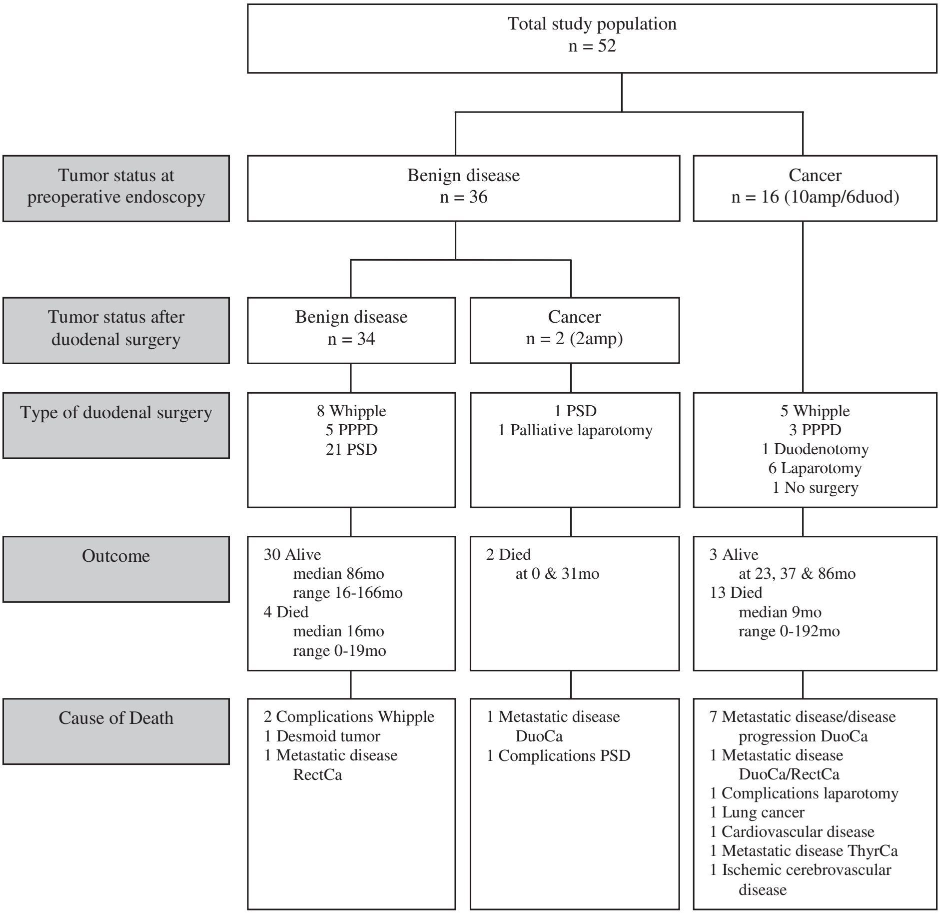

Figure 1. Flow diagram: tumor status at preoperative endoscopy, tumor status after duodenal surgery, type of duodenal surgery performed, outcome, and cause of death. *Abbreviations*: amp, ampullary; duod, duodenal; Whipple, classical Whipple's pancreaticoduodenectomy; PPPD, pylorus preserving pancreaticoduodenectomy; PSD, pancreas-sparing duodenectomy; RectCa, rectal cancer; DuoCa, duodenal cancer; ThyrCa, cancer of thyroid gland.

duodenal adenomatosis, including duodenotomy with polypectomy (n=2) and ampullectomy with bile and pancreatic duct reconstruction (n=1).

Two patients (6%) with preoperative benign duodenal adenomatosis were diagnosed with cancer after a previous operation, including 1 ampullary carcinoma found at postoperative histopathology and 1 locally advanced ampullary tumor with liver metastasis found during laparotomy.

Patients with duodenal cancer at preoperative endoscopy

The characteristics of 16 patients with duodenal cancer diagnosed at preoperative endoscopy are included in ***Table 3***. Six of 16 patients were under surveillance: in 2, cancer was identified

Table 3. Characteristics of eighteen patients with duodenal cancer.

		Mutation		Primary CR surgery			Duodenal cancer and surgery	
Case no	Sex	Exon	Codon	Type	Age (yr)	CRC	Mode of presentation	Site
1[(1)]	F	15	1209	Ileostomy	27	-	Surveillance (Spigelman IV)	A
2[(1)]	F	5	178	IRA	29	-	Surveillance (Spigelman IV)	A
3	F	15	685	IRA	33	-	Cholestasis	A
4	M	nk	nk	Ileostomy	29	Rectum	Anaemia, syncope, melaena	D
5	F	11	499	Ileostomy	63	Colon	Cholestasis	A
6	M	11	516	IPAA	40	-	nk	A
7	M	nk	nk	IRA	40	-	Anaemia, vomiting, weight loss	D
8	F	15	728	IRA	17	-	Jaundice	A
9	M	14	630	IRA	32	-	Abdominal pain	D
10	F	4	150	IRA	42	Colon	Jaundice, vomiting	A
11	M	13	554	IRA	25	-	Surveillance (cancer)	A
12	M	13	554	IRA	25	-	Surveillance (cancer)	A
13	F	12	541	IRA	36	Colon	Jaundice, anaemia	A
14	F	14	622	IRA	22	-	Abdominal pain	D
15	M	nk	nk	IRA	15	-	First surveillance endoscopy	D
16	M	13	564	IPAA	18	Colon	Jaundice	A
17	F	15	1464	Ileostomy	40	Colon, Rectum	Anaemia, weight loss	A
18	F	15	1062	Ileostomy	36	Colon, Rectum	Abdominal pain	D

[(1)] Patients who underwent prophylactic duodenectomy, but were diagnosed with cancer after surgery.
[(2)] Patient died 4 months after diagnosis at endoscopy and radiologic assessment of irresectable ampullary cancer.
[(3)] No material for histopathological evaluation was obtained during duodenal surgery, TNM-classification based on histopathological examination of biopsy material from preoperative endoscopy, clinical observations and/ or additional radiologic evaluation.

by endoscopic surveillance, and 4 presented with signs or symptoms of disease. Two patients presented with symptoms 12 and 22 months after scheduled endoscopic surveillance, when their duodenal adenomatosis was graded as Spigelman III and IV disease, respectively. In the latter patient, the recommended surveillance interval was not followed because of additional morbidity. The other 2 symptomatic patients who underwent duodenotomy with ampullectomy and palliative laparotomy, respectively, previously were not considered candidates for prophylactic surgery because of a desmoids tumor and severe jejunal adenomatosis.

Ten of 16 patients were not under surveillance and had no upper gastrointestinal endoscopy performed for at least the previous 5 years: 8 patients presented symptomatically, 1 patient underwent his first surveillance endoscopy, and for 1 patient the mode of presentation was unknown.

Duodenal cancer and surgery *(continued)*				
TNM	**Type of surgery**	**Age (yr)**	**Survival (mo)**	**Cause of death**
HGD, TxNxM1[3]	Lapar, no intervention	59	31	Metastatic disease, liver
T1NxMx	PSD + antrum resection	60	0	Complications of surgery: multi-organ failure
T1NxMx	Whipple	57	Alive 86	-
T3NxM1	Lapar, GJS	62	9	Progression of disease, peritonitis carcinomatosis
T3NxMx	Whipple	67	133	Metastatic disease from cancer of thyroid gland
T3N1Mx	PPPD	48	15	Metastatic disease
T4NxM1[3]	Lapar, GJS	57	9	Metastatic disease, retroperitoneum and psoas
TxN1M1[3]	Lapar, GJS	32	8	Metastatic disease, liver
T2N1Mx	Whipple	58	48	Lung cancer
TxNxMx[3]	No surgery	53	4[2]	Progression of disease
T3N1Mx	PPPD	53	76	Cardiovascular disease
T1N0M0[3]	Duodenotomy with ampullectomy	59	Alive 37	-
TxNxMx	Lapar, placement of billiary T-drain	47	3	Progression of disease
T4NxM1	Lapar, repositioning of intra-abdominal herniation	48	7	Metastatic disease, liver and lung
T2NxMx	PPPD	49	192	Ischemic cerebrovascular disease
T1N0Mx	Whipple	39	Alive 23	-
T4N0Mx	Whipple	40	11	Metastatic disease from synchronous CR and duodenal cancer
TxN1M1	Lapar, GJS, partial resection of small bowel	56	0	Complications of surgery: sepsis, bile leakage, bleeding

Abbreviations: CR, colorectal; CRC, colorectal cancer; IRA, Ileorectal anastomosis; Ileostomy, Proctocolectomy with ileostomy; IPAA, Ileal pouch-anal anastomosis; A, Ampullary; D, Duodenal; TNM, Tumor Node Metastasis classification; HGD, High grade dysplasia; PSD, pancreas-sparing duodenectomy; Whipple, classical Whipple's pancreaticoduodenectomy; PPPD, pylorus preserving pancreaticoduodenectomy; Lapar, laparotomy; GJS, gastrojejunostomy; nk, not known.

Twelve patients presented with clinical signs or symptoms of duodenal cancer, including jaundice and/or cholestasis (n=6), anemia (n=4), abdominal pain (n=3), vomiting (n=2), weight loss (n=2), syncope (n=1), and melena (n=1). All patients presenting with jaundice and/or cholestasis had ampullary cancer, whereas all cases presenting with abdominal pain had duodenal cancer.

Complications after duodenectomy

Postoperative mortality and morbidity was evaluated for all patients who underwent duodenectomy (n=43, 35 for benign disease, 8 for duodenal cancer). Overall 30-day mortality was 5% (n=2). One patient died as the result of pericarditis with multiorgan failure 12 days after PSD for Spigelman IV disease histopathology: ampullary cancer), and one had an anastomotic

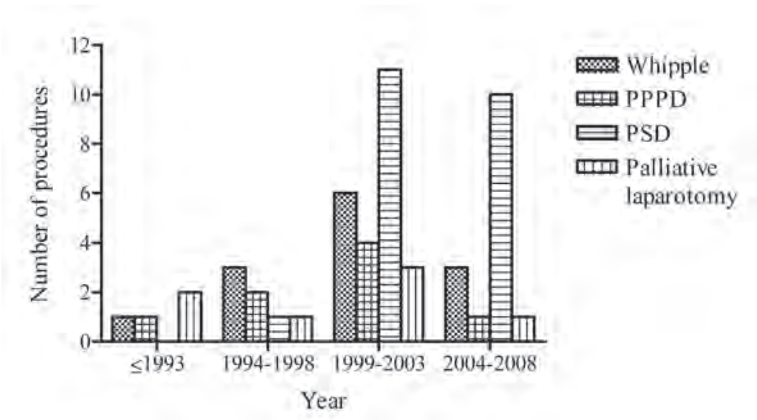

Figure 2. Number and type of surgical procedures performed in five year periods. *Abbreviations*: Whipple, classical Whipple's pancreaticoduodenectomy; PPPD, pylorus preserving pancreaticoduodenectomy; PSD, pancreas-sparing duodenectomy.

leak with abdominal sepsis and died 19 days after Whipple for Spigelman IV disease. A third patient died 7 months after undergoing the Whipple procedure for Spigelman IV disease. The postoperative course was complicated by necrotizing pancreatitis, enterocutaneous fistula formation, and thrombosis of the superior caval vein. In all 3 cases of mortality, patients were surgically treated for what was considered to be benign duodenal disease.

An overview of all postoperative complications is shown in ***Table 4***. A total of 33 in-hospital complications in 21 patients (49%; 17 benign disease, 4 cancer) were reported. There was no significant difference found in number of patients experiencing in-hospital complications when we compared patients with benign disease and patients with cancer at preoperative endoscopy (Fisher's exact test, p=.62), and when we compared the 3 types of duodenectomy (Fisher's exact test, p=.20).

Seven patients (16%; 5 benign disease, 2 cancer) needed unplanned relaparotomy because of intra-abdominal infection (n=3), hemorrhage (n=2), or anastomotic leakage (n=1). In 1 patient, no abnormalities were found, and the patient was treated for pancreatitis. A total of 10 long-term complications in 8 patients (19%; seven benign disease, 4 cancer) were reported, including 1 patient who previously suffered in-hospital morbidity.

Survival and causes of death

As shown in ***Figure 1***, 4 of 36 patients (11%) with benign duodenal disease at endoscopy died of causes related to their duodenal disease, including metastatic duodenal cancer (n=1) and postoperative mortality after duodenectomy (n=3); 2 other patients (6%) died of unrelated causes.

Nine of 16 patients (56%) diagnosed with cancer at preoperative endoscopy died of causes related to duodenal cancer, including metastatic disease or progression of disease (n=7), metastatic disease originating from either duodenal or synchronous rectal and colonic cancer (n=1), and postoperative morbidity after palliative laparotomy (n=1). Four patients (25%) died of unrelated causes, and 3 patients (19%) were alive at time of study closure.

Table 4. Patients with complications after duodenectomy for benign duodenal disease or cancer at preoperative endoscopy.

Group	n	Total morbidity	In-hospital morbidity[(1)]	Long-term morbidity[(2)]
Benign disease at endoscopy	35	23[(3)] (66%)	17[(3)] (49%)	7[(3)] (20%)
Whipple	8	6	5	1
PPPD	5	3[(3)]	3[(3)]	1[(3)]
PSD	22	14	9	5
Cancer at endoscopy	8	5 (63%)	4 (50%)	1 (13%)
Whipple	5	4	4	0
PPPD	3	1	0	1
Overall	43	28 (65%)	21 (49%)	8 (19%)

[(1)] In-hospital morbidity: intra-abdominal abscess (n=6), fistula formation (n=5), anastomotic leakage (n=6), pancreatitis (n=2), sepsis (n=4), postoperative hemorrhage (n=4), surgical site infection (n=2), trombo-embolism of superior caval vein (n=2, in 1 case with pulmonary embolism), occlusion of branches of the hepatic artery with local ischemia of liver tissue adjacent to the falciform ligament (n=1), and pericarditis with multi-organ failure (n=1).
[(2)] Long-term morbidity: (chronic) pancreatitis (n=3), incisional hernia (n=1), stenosis of the enterobiliary anastomosis (n=2, in 1 case with cholangitis), cutanous fistula and abscess formation resulting in sepsis induced by migrated mesh into the jejunum (n=1), anastomotic erosions/ulcer (n=3).
[(3)] Includes one patient with both in-hospital and long-term morbidity.
Of note: one patient can have more than one complication.
Abbreviations: Whipple, classical Whipple's pancreaticoduodenectomy; PPPD, pylorus preserving pancreaticoduodenectomy; PSD, pancreas-sparing duodenectomy.

The observed differences in distribution of causes of death for patients with benign duodenal disease or cancer at preoperative endoscopy, as either related or unrelated to duodenal disease, were not significant (Fisher's exact test, p=.14). Survival of patients with cancer at preoperative endoscopy was less than the survival of patients who underwent prophylactic surgical resection for benign disease (***Figure 3***; log-rank test, p<.001).

When considering all 18 patients with cancer, the prognosis of duodenal cancer was deemed poor; the Kaplan-Meier estimated median survival was 11 months. There was a difference in estimated median survival of patients who underwent duodenectomy and patients who underwent palliative intervention (76 vs 8 months, log-rank test, p<.05). Estimated median survival rates for cancer patients under surveillance versus cancer patients not under surveillance were 9 and 11 months, respectively (log-rank test, p=.54). The difference in estimated median survival after duodenal surgery between patients with ampullary cancer and duodenal cancer (15 vs 9 months, respectively) was not significant (log-rank test, p=.77).

Recurrence

Data on endoscopic follow-up were available in 32 patients. Adenomas were seen during follow-up endoscopy in 18 patients. Four patients (13%) with adenomas at the first postoperative follow-up endoscopy were excluded because these adenomas might have been present before duodenectomy was performed. Hence, in 14 of 28 patients (50%) adenomas recurred with a Kaplan-Meier estimated median time from surgery to recurrence of 75 months. Recurrence of adenomas was seen in 3 of 7 patients after Whipple (43%), 4 of 6 after PPPD (67%), and 7 of 15 after PSD (47%), with estimated

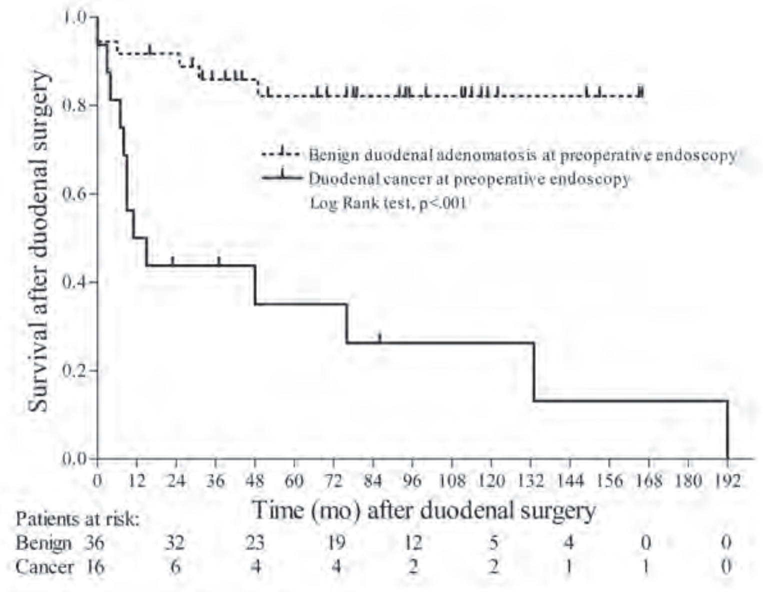

Figure 3. Kaplan-Meier curve: survival after duodenal surgery stratified for tumor status at preoperative endoscopy: benign duodenal adenomatosis or duodenal cancer. (Duodenal surgery includes: classical Whipple's pancreaticoduodenectomy; pylorus preserving pancreaticoduodenectomy; pancreas-sparing duodenectomy; laparotomy with or without palliative intervention, and duodenotomy with ampullectomy.) Of note: in one cancer patient no duodenal surgery was performed and survival (4 months) was measured from diagnostic endoscopy.

median time from surgery to recurrence of 103, 53, and 66 months, respectively (log rank test, p=.28). The median score in points by Spigelman stage at recurrence was 5 (range, 3-7), with 7 cases of Spigelman I, 6 cases of Spigelman II, and 1 case of Spigelman III disease. Onepatient was diagnosed with cancer at the hepaticojejunostomy 156 months after PPPD for duodenal cancer.

DISCUSSION

In the present study, 18 patients of 1,066 patients with FAP in the Dutch registry (1.7%) were diagnosed with duodenal cancer between the years 1975 and 2008. Recently, a comparable rate of 1.9% was reported on the basis of 20 cases of cancer diagnosed in the St. Mark Hospital Polyposis Registry between 1969 and 2005.[16] Both studies suggest that the prevalence of duodenal cancer might be less than the 4.5% reported in the late 1980s.[11] It is unclear whether this difference in prevalence reflects a true decrease in duodenal cancer prevalence that subsequently might be attributed to improved management of duodenal disease in patients with FAP.

Our results support previous findings on the poor prognosis of duodenal cancer in patients with FAP.[11,14-16] Notably, in almost one-half of the patients presenting with duodenal cancer at endoscopy, the cancer stage was too advanced to perform a curative resection. The aim of surveillance programs is to identify patients with advanced duodenal adenomatosis before cancer develops. In the majority of our cancer cases, either no surveillance was performed or the recommendation on surveillance interval was not followed. In contrast, nearly all patients with benign disease were under surveillance. The survival of patients who underwent prophylactic duodenectomy was far better. Our findings imply that if appropriate surveillance intervals were followed, nearly one-half of the cancers could have been diagnosed at a treatable stage or could even have been prevented by timely prophylactic intervention. Interestingly, patients with duodenal cancer had been diagnosed with colorectal cancer at previous initial colorectal surgery more often compared with patients with benign disease. All patients with FAP, but particularly patients diagnosed with cancer previously at initial colorectal surgery, should be motivated to follow strictly the recommended surveillance intervals.

Notwithstanding this recommendation, limitations in the sensitivity of endoscopic surveillance should be kept in mind. Two patients (6%) who underwent surgery for benign duodenal adenomatosis were diagnosed with cancer on the basis of histopathologic examination of the resected tissue. This finding illustrates that the presence of cancer may be underestimated by taking endoscopic biopsies of adenomas, most probably because of sampling error and the small size of the biopsies taken.[30,31] Moreover, although the Spigelman classification has been shown to correlate with the risk of developing cancer[17], it seems inadequate to assess the individual patient's risk of cancer accurately. The classification focuses primarily on nonampullary duodenal disease, and the evaluation of ampullary disease should be taken into account separately.[16] In patients with advanced duodenal adenomatosis, endoscopic ultrasonography may provide additional information on malignant invasion.[32,33] Fludeoxyglucose positron emission tomography (FDG-PET) has been shown to differentiate between adenomas and carcinomas, detecting all cancer cases in patients with FAP with duodenal adenomas.[34] Although the role of FDG-PET at present has not been established firmly, it represents a promising modality in guiding treatment decisions concerning duodenectomy that warrants further attention.

Ideally, a prophylactic procedure should carry no risk of death and have low morbidity while preventing future disease. The 30 patients alive after extensive duodenectomy for benign duodenal adenomatosis could be considered as beneficiaries of prophylactic duodenectomy. Overall postoperative morbidity and mortality, however, is substantial. All 3 cases or mortality in our series occurred in procedures that were intended as prophylactic. In addition, postoperative in-hospital morbidity occurred in one-half of the patients, either after prophylactic resection or cancer treatment. In previous studies authors have revealed comparable rates of postoperative morbidity of 38-60% and mortality of 0-12% after duodenectomy (PSD and PPPD) in patients with FAP.[25,35-37] It has been suggested that patients with FAP might be at greater risk of complications compared with patients without FAP because of more demanding operative conditions related to more technically demanding anastomotic reconstruction of a soft pancreas with a nondilated biliary and pancreatic ductal system, and adhesions caused by previous colorectal surgery.[25,37] Furthermore, a preoperatively undetected nonfusion of the pancreatic duct (pancreas divisum) was the cause of postoperative complications in patients with FAP after PSD.[25,38] To prevent these complications, magnetic resonance cholangiopancreatography might be indicated in standard preoperative evaluation for PSD.

Recurrence of adenomas in the reconstructed proximal small bowel after duodenectomy occurred in one-half of all patients with available postoperative endoscopic follow-up data within just more than 6 years, and 1 patient developed cancer after PPPD at the hepaticojejunostomy. These findings are in line with previous reports of recurrence of adenomas after extensive surgery[15,39] and malignant degeneration in residual duodenal mucosa after duodenectomy.[28] As shown by our finding of 13% of patients with adenomas at the first postoperative endoscopy, adenomas may already be present in the jejunum used for construction, especially in patients with more advanced duodenal adenomatosis.[40] To avoid this possibility, preoperative inspection of the jejunum by single- or double-balloon enteroscopy is advised. Our findings support the recommendation that upper gastrointestinal surveillance should be continued after duodenectomy, because the risk of cancer is not entirely eliminated.[28,35,36,39]

The strength of our study is that we reviewed data covering the total Dutch population of patients with FAP who were receiving medical care in both regional hospitals as well as academic referral centers. We evaluated a considerably large cohort of patients with extensive follow-up, including not only patients with duodenal cancer, but also patients who underwent prophylactic duodenectomy for advanced duodenal adenomatosis. The number of cancer cases involved, however, is small, and power might therefore be inadequate to prove differences to be statistically significant. Study limitations include the retrospective study design, resulting possibly in a cohort effect. Missing data, although limited, might have biased the results, particularly where it concerns clinical follow-up data, resulting in less reported long-term postoperative morbidity rate and adenoma recurrence.

In conclusion, our study illustrates the poor prognosis of duodenal cancer, justifying in our opinion an aggressive prophylactic surgical approach to advanced benign duodenal disease, despite the substantial risk of morbidity and mortality. Strict adherence to the recommended surveillance intervals is essential for well-timed operative intervention. Treatment decisions are to be made by critically evaluating a patient's individual characteristics, taking into account age, history of colorectal cancer, previous abdominal surgery, course of the Spigelman classification over time, previous treatment of duodenal adenomatosis, and additional morbidity. Even after radical duodenal resection, patients have to bear the continuing burden of endoscopic surveillance and threat of cancer.

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CHAPTER 3

Management of sporadic duodenal adenomas and the association with colorectal neoplasms: A retrospective cohort study

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PUBLISHED IN: *JOURNAL OF CLINICAL GASTROENTEROLOGY*, 2012, 46(5): 390-6.

ABSTRACT

With the widespread use of esophagogastroduodenoscopy, an increasing number of sporadic duodenal adenomas are diagnosed. An optimal algorithm for management has not been fully defined. Accumulating data suggest an association with colorectal neoplasms. Aim of this study was to evaluate management, outcome, and follow-up of patients with sporadic duodenal adenomas and assess the presence of colorectal neoplasms. Patients diagnosed with sporadic duodenal adenomas at our institute from 1986 until 2008 were retrospectively reviewed. Data were collected from medical records. Fifty-four patients (28 men, 52%) were diagnosed with a sporadic duodenal adenoma at a median age of 59 years (range, 27 to 84y); 33 patients (61%) underwent endoscopic or surgical intervention, 5 (9%) were only followed endoscopically, and 16 (30%) underwent no intervention or follow-up. Complete endoscopic removal was accomplished in at least 81% of cases, and no complications were reported; surgical intervention was complicated in 4 patients, with 1 resulting in death. Adenoma recurrence was 20% at a median follow-up of 18 months (range, 4 to 54mo), but no carcinoma developed. Colorectal neoplasms were found in 16 of 29 patients (55%) who underwent colonoscopy, including 2 cancers (7%), 7 advanced adenomas (24%), and 7 nonadvanced adenomas (24%). In conclusion, although no consistent approach to management of sporadic duodenal adenomas was followed, no duodenal carcinoma developed during the follow-up. Endoscopic intervention is preferred over surgical intervention, whenever possible. Once complete removal is ascertained, there is no strict indication for regular follow-up esophagogastroduodenoscopy, especially in elderly patients or patients with relevant comorbidity. Colonoscopic assessment is warranted in all patients diagnosed with sporadic duodenal adenomas.

Keywords: sporadic duodenal adenomas; colorectal neoplasms; management; recurrence; follow-up

Abbreviations: FAP, familial adenomatous polyposis; EGD, esophagogastroduodenoscopy; EMR, endoscopic mucosal resection; GI, gastrointestinal; PD, pancreaticoduodenectomy

Although duodenal adenomas are common lesions in patients with familial adenomatous polyposis (FAP) and attenuated FAP,[1] sporadic duodenal adenomas are rare. With the widespread use of esophagogastroduodenoscopy (EGD) for evaluation of upper gastrointestinal (GI) complaints, the number of patients found to have duodenal lesions has increased.[2-6] The prevalence of sporadic duodenal polyps during routine EGD was reported to range from 0.3% to 4.6%, but only 7% of the polyps were dysplastic lesions or adenomas.[6] In accordance with the colorectal adenoma-to-carcinoma sequence, these small bowel adenomas are regarded as noninvasive premalignant lesions. This is supported by the findings of occurrence of adenocarcinoma within small bowel adenomas and the presence of residual adenomatous mucosa adjacent to or within most carcinomas.[7,8]

It is generally recommended to resect these lesions, with local duodenotomy or radical pancreaticoduodenectomy (PD) being the most common method of resection.[9] More recently, several studies on endoscopic management of duodenal adenomas, including endoscopic mucosal resection (EMR) and endoscopic snare polypectomy or ampullectomy, report encouraging results.[2,10-16]

However, an optimal algorithm for treatment and follow-up of patients with sporadic duodenal adenomas has not yet been fully defined.[17] In addition, there are accumulating data that support a clinically relevant association between sporadic duodenal adenomas and colorectal neoplasms, and it has been stated that patients with sporadic duodenal adenomas should therefore be screened by performing colonoscopy.[18-25]

To optimize patients' management, the aim of this retrospective study was to evaluate the management of the sporadic duodenal adenomas, treatment outcome, and follow-up. In addition, we assessed the prevalence of colorectal adenomas and their pathologic features in patients with sporadic duodenal adenomas.

PATIENTS AND METHODS

This study was conducted according to International Conference on Harmonization Good Clinical Practice and complied to the principles of the amended declaration of Helsinki and Dutch legislation. The electronic database of the Department of Pathology at the tertiary referral Hospital Radboud University Nijmegen Medical Centre was retrospectively reviewed to identify all patients diagnosed with a duodenal adenoma in the time period from January 1st 1986 until December 31st 2008. Patients diagnosed with familial polyposis syndromes were not included.

Medical records, both on paper and electronic, were reviewed for patient characteristics. Data on features of duodenal adenoma were collected from EGD and histopathology reports, including endoscopic size and location, degree of dysplasia, and histologic type. When degree of dysplasia was scored as "intermediate" (22 of 54 cases), tissue sections were revised and classified as low-grade or high-grade dysplasia by an expert pathologist (IDN) according to the Vienna classification.[26,27] When histologic type was not reported (12 of 54 cases), tissue sections were reevaluated by the same expert pathologist.

Type of endoscopic and surgical intervention for duodenal adenomas and any complication were recorded. Total follow-up time was defined as the time interval from EGD on which the adenoma was diagnosed to the last EGD performed. Follow-up data were analyzed until study closure, December 31st 2010. Recurrence was defined as identification of an adenoma at the same localization of a previously successfully removed adenoma or at a different localization in the duodenum.

Finally, endoscopic and histopathological features of premalignant lesions found during any colonoscopic assessments performed in patient's medical history, before or after the duodenal adenoma was diagnosed, were recorded. When multiple lesions were found, the most advanced colorectal lesion was considered.

Advanced adenomas (duodenal and colorectal) were defined as: size ≥10 mm, a villous component, and/or high-grade dysplasia. When endoscopic size was not specified, classification was solely on the basis of histopathologic features.

Patients were classified in 3 groups: (1) "Intervention": patients who underwent either endoscopic or surgical intervention; (2) "Wait and see": patients who were followed endoscopically; and (3) "No intervention or follow-up."

Statistical analysis

Statistical analysis was performed using SPSS statistical software version 16.0 (SPSS, Chicago, IL). Frequency tables were provided for description of patients and adenoma characteristics. Patient and adenoma characteristics were described by use of median and range for continuous variables and percentages for categorical variables. Group comparison was performed on the basis of the classification "Intervention" versus "Wait and see" versus "No intervention/follow-up." Differences in age at diagnosis were examined using Kruskal-Wallis test. Pearson χ^2 test was used to compare discrete variables. Fisher exact test was used where appropriate. Survival data were analyzed by Kaplan-Meier survival analysis and log rank test. P-values <0.05 (2-sided) were considered statistically significant.

RESULTS

Patient and adenoma characteristics

Fifty-four patients (28 men, 52%) with sporadic duodenal adenomas were identified, with a median age at diagnosis of 59 years (range, 27 to 84y). Characteristics of the patients and the adenoma diagnosed are shown in ***Table 1***, for the total study population and the three predefined groups. Characteristics of treatment, follow-up, recurrence, and survival are shown in ***Figure 1***. Indications for EGD were the following: abdominal pain (n=17, 31%), anemia (n=10, 19%), follow-up for esophageal or gastric lesions (n=9, 17%), weight loss (n=6, 11%), dysphagia (n=4, 7%), symptoms of reflux disease (n=3, 6%), upper GI bleeding (n=2, 4%), follow-up for peptic ulcer disease (n=1, 2%), diagnostic workup for liver metastases with unknown primary cancer (n=1, 2%), and unknown indication (n=1, 2%).

Table 1. Patient and duodenal adenoma characteristics for the overall study population and predefined subgroups 'Intervention group', 'Wait and see group', and 'No intervention/follow-up group'.

		Total		Intervention		Wait and see		No intervention/ follow-up		p-value
Patients		**54**		**33**	**(61%)**	**5**	**(9%)**	**16**	**(30%)**	
Gender	Male	28	(52%)	15	(45%)	4	(80%)	9	(56%)	.389[1]
	Female	26	(48%)	18	(55%)	1	(20%)	7	(44%)	
Age (yrs, median [range])		60	[27-85]	58	[27-84]	69	[40-74]	60	[46-83]	.302[2]
Duodenal adenomas										
Localisation	Bulb	17	(32%)	9	(27%)	2	(40%)	6	(38%)	.522[1]
	Post bulbar	22	(41%)	17	(52%)	1	(20%)	4	(25%)	
	Multiple	4	(7%)	2	(6%)	1	(20%)	1	(6%)	
	Ampulla	5	(9%)	4	(12%)	0	(0%)	1	(6%)	
	Not specified	6	(11%)	1	(3%)	1	(20%)	4	(25%)	
Endoscopic size	<5mm	18	(33%)	11	(33%)	1	(20%)	6	(38%)	.068[1]
	≥5, <10mm	6	(11%)	2	(6%)	0	(0%)	4	(25%)	
	≥10mm	13	(24%)	9	(27%)	3	(60%)	1	(6%)	
	Not specified	17	(31%)	11	(33%)	1	(20%)	5	(31%)	
Dysplasia	Low grade	47	(87%)	26	(79%)	5	(100%)	16	(100%)	.089[1]
	High grade	7	(13%)	7	(21%)	0	(0%)	0	(0%)	
Histological type	Tubular	26	(48%)	14	(42%)	2	(40%)	10	(63%)	.697[1]
	Tubulovillous	17	(31%)	12	(36%)	2	(40%)	3	(19%)	
	Villous	11	(20%)	7	(21%)	1	(20%)	3	(19%)	
Non-advanced/ advanced	Non-advanced	21	(39%)	10	(30%)	1	(20%)	10	(63%)	.061[1]
	Advanced	33	(61%)	23	(70%)	4	(80%)	6	(38%)	

[1] Pearson's Chi-square/Fisher's exact test test.
[2] Kruskal Wallis test.
p-values of <0.05 (two-sided) were considered statistically significant. Advanced adenomas were defined as: size ≥10mm, a villous component, and/or high-grade dysplasia.

During the time period reviewed, the mean number of diagnostic EGDs performed at our Department of Gastroenterology and Hepatology each year was approximately constant, on an average 1500 procedures each year. The increase in number of sporadic duodenal adenomas diagnosed per 4-year time interval is visualized in ***Figure 2***. In the recent years from 2005 to 2008, 11 of 19 adenomas (58%) were small adenomas (<5 mm); in the years before 2005, only 7 of the 18 adenomas (39%), of which size was specified, were small (χ^2, p=0.248).

At study closure, 21 of 54 patients (39%) had died. Median age at death was 69 years (range, 56 to 92y). Overall estimated median of survival after diagnosis of sporadic duodenal adenoma was 13 years (Kaplan-Meier, 95% confidence interval, 4-22y). Survival differed significantly between

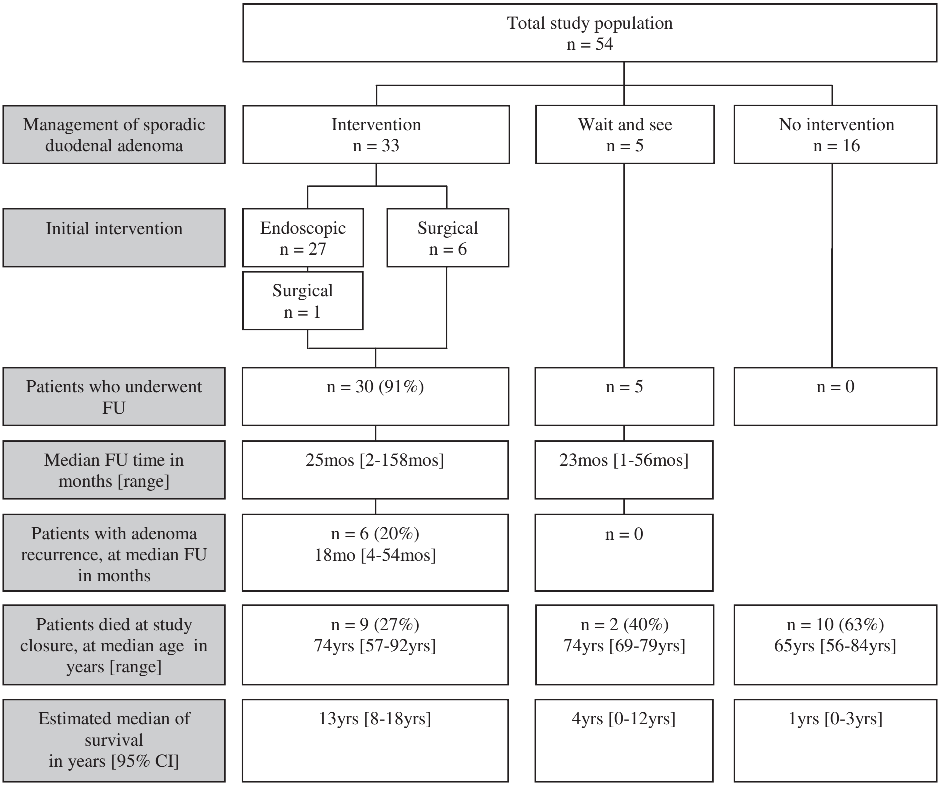


Figure 1. Management and outcome of patients diagnosed with duodenal adenoma. *Abbreviations*: CI, confidence interval; FU, follow-up.

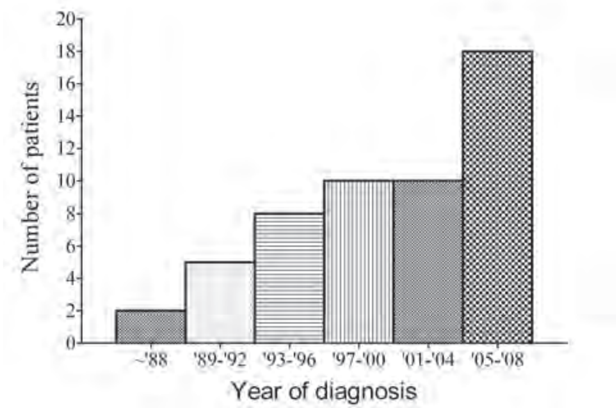
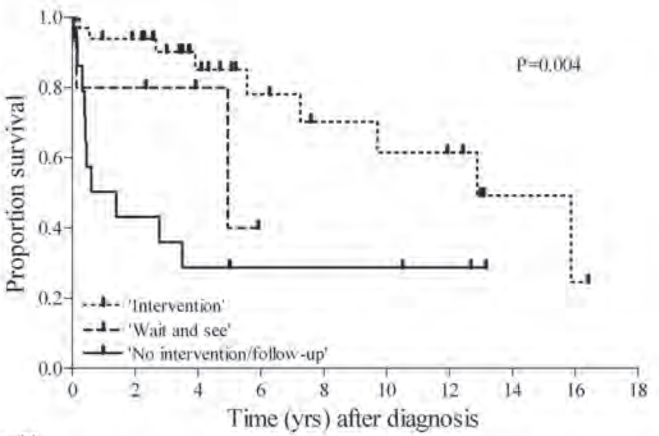


Figure 2. Number of patients diagnosed with a sporadic duodenal adenomas per 4 year period at the Radboud University Nijmegen Medical Centre.



Patients at risk										
'Intervention'	33	29	17	11	8	7	6	2	1	0
'Wait and see'	5	4	2	0	0	0	0	0	0	0
'No intervention'	16	6	4	3	3	3	2	0	0	0

Figure 3. Survival after diagnosis of sporadic duodenal adenoma.

the 3 groups (log rank, p=0.004, ***Figure 3***) mainly between the "Intervention group" and "No intervention/follow-up group" (log rank, p=0.001). Causes of death were not related to the duodenal adenoma, except for 1 patient who died of complications after a Whipple procedure.

Intervention group

In 33 of 54 patients (61%), with a median age at diagnosis of 58 years (range, 27 to 84y), an intervention for the sporadic adenoma was performed.

In 27 patients (50%) initial therapy was endoscopic intervention, including removal by taking biopsies (n=18), argon plasma coagulation (n=5), and snare polypectomy (n=4). In 22 patients (81%), complete endoscopic removal of the adenoma was accomplished, in 1 treatment session in 20 cases, in 3 sessions in 1 case, and in 4 sessions in 1 case. Three patients in whom complete removal was not accomplished in 1 session were still under treatment at study closure. In 2 patients complete endoscopic removal was not accomplished; 1 patient needed surgery, and in 1 patient further treatment and follow-up was terminated because of comorbidity (mental retardation). No complications were reported after any of the endoscopic interventions.

In 6 patients (11%), initial therapy was surgical intervention in all cases for an advanced duodenal adenoma. Factors that were considered as decisive to perform surgery included signs of active bleeding (n=1), large size of the adenoma (n=3), presence of high-grade dysplasia (n=3), and/or ampullary localization (n=2). One patient underwent surgical intervention after failed initial treatment by snare polypectomy of a large ampullary adenoma with high-grade dysplasia. In total, 7 surgical procedures were performed: 3 duodenotomies with polypectomy/ampullectomy, 2 partial duodenectomies, 1 pylorus sparing PD, and 1 classical Whipple PD (Whipple). Histopathological examination of the resected specimen did not reveal cancer in any of these cases. Complications after surgical interventions were reported in 4 of 7 cases (57%), including 2 cases after duodenotomy (liver abscess, anemia/pneumonia), 1 after PD (anastomotic leak and pancreatic fistula with sepsis), and 1 after Whipple (necrotizing pancreatitis and abdominal infection with subsequent multiple organ failure resulting in death 25 days after surgery).

Endoscopic follow-up was performed in 30 of the 33 patients (91%) who underwent intervention, with a median follow-up of 25 months (range, 2 to 158mo). Duodenal adenoma recurred in 6 of 30 patients (20%) who underwent follow up at a median of 18 months (range, 4 to 54mo), an advanced adenoma in 3 patients, and a nonadvanced in 3 patients. None of the patients developed duodenal cancer. No endoscopic follow-up was performed in 3 of the 33 patients (9%) who underwent an intervention, including the patient who died of complications after Whipple, 1 patient with comorbidity (mental retardation), and 1 patient for whom no follow-up was planned.

Wait and see group

Five of 54 patients (9%), with a median age at diagnosis of 69 years (range, 40 to 74y), did not undergo an endoscopic or surgical intervention, but were followed endoscopically. Specific motivation for the choice of "Wait and see" could not be identified from the medical records in each of these cases. Decisive factors that were mentioned included a high age at diagnosis and too much comorbidity to undergo surgical intervention for adenomas with low-grade dysplasia that were too large to resect endoscopically. Median follow-up was 23 months (range, 1 to 56mo). No changes in size or histopathological features were reported during follow-up, and no additional duodenal adenomas were found.

No intervention/follow-up group

Sixteen of 54 patients (30%) with a median age at diagnosis of 60 years (range, 46 to 83y) underwent no therapeutic intervention or endoscopic follow-up, including 6 patients (38%) with an advanced duodenal adenoma. Particularly in this group, significant comorbidity was noted, including local progression or metastatic disease of cancer (kidney, lung, pancreas, esophagus, mamma, and larynx), mental retardation, Morbus Steinert, chronic obstructive pulmonary disease with chronic heart failure, and liver cirrhosis. Because of comorbidity, in over half of patients, life expectancy was limited at time of diagnosis of duodenal adenoma.

Colonoscopy findings

In 29 of 54 patients (54%), a colonoscopy was performed at any time in patient's medical history, as shown in ***Table 2***. Colorectal neoplasms were found in 16 of 29 patients (55%). Two patients (7%) were diagnosed with colorectal cancer: 1 with a carcinoma in the transverse colon (tumor, node, metastasis stage unknown) and 1 with an adenocarcinoma in the cecum (T3N1M0). Cancers were diagnosed 7 years before and 10 years after diagnosis of duodenal adenoma, respectively. Another 14 patients (48%) had at least 1 colorectal adenoma, including 7 patients (27%) with an advanced adenoma. In 1 patient the (nonadvanced) adenoma was localized in the right hemicolon, in all other patients adenomas were localized in the left hemicolon. We found

Table 2. Colonoscopic findings in patients with duodenal adenomas (total group and non-advanced vs. advanced duodenal adenomas).

	Duodenal adenomas						
	Total		Non-advanced		Advanced		p-value
Number of patients	54		21		33		
Number of patients who underwent colonoscopy	29	(54%)	11	(52%)	18	(55%)	1.000[(1)]
Colorectal neoplasm found							
All colorectal neoplasms	16	(55%)	5	(45%)	11	(61%)	.466[(1)]
Cancer or advanced adenoma	9	(31%)	2	(18%)	7	(39%)	1.000[(1)]
Cancer	2	(7%)	0	(0%)	2	(11%)	
Advanced adenoma	7	(24%)	2	(18%)	5	(28%)	
Non-advanced adenoma	7	(24%)	3	(27%)	4	(22%)	

[(1)] Pearson's χ^2/Fisher's exact test test.
p-values of <0.05 (two-sided) were considered statistically significant. Advanced adenomas (duodenal and colorectal) were defined as: size ≥10mm, a villous component, and/or high-grade dysplasia.

no significant differences in number of patients with colorectal neoplasms (nonadvanced adenoma, advanced adenoma, or cancer) comparing patients with nonadvanced or advanced duodenal adenomas (χ^2/Fisher exact, p>0.05; ***Table 2***). First diagnosis of a colorectal neoplasm (not necessarily the most advanced lesion) was at a median of 4 months before diagnosis of sporadic duodenal adenoma with a wide range of 20 years before and 10 years after diagnosis of sporadic duodenal adenoma. The diagnosis of a sporadic duodenal adenoma was specifically stated as indication for colonoscopy in only 8 patients (15%). In these cases, colonoscopy was performed at a median of 6 months (range, 0 to 25mo) after diagnosis. In a ninth patient, with extensive comorbidity, presence of colorectal neoplastic lesions was examined by doublecontrast barium enema 3 months after diagnosis. In 4 of these 9 patients (44%), colorectal adenomas were found, of which 1 was an advanced adenoma.

DISCUSSION

Duodenal adenomas are increasingly encountered, which is explained by the widespread use of EGD in patients with upper GI complaints.[2-6] We also found an increase in incidence of duodenal adenomas over the past decades, whereas the number of performed EGDs remained constant. Improved endoscopic techniques that enhance visualization of smaller lesions might explain the increase in incidence, as illustrated by our observation of an (not significant) increase in proportion of smaller adenomas in more recent years.

Abdominal pain and anemia were the most common indications for EGD. These and a variety of other nonspecific symptoms, including esophageal reflux, nausea and vomiting, dyspepsia, and GI bleeding, were previously reported as indication for EGD on which adenomas were found.[12,14,18,22] In studies focusing on ampullary adenomas, jaundice and pancreatitis were also common symptoms at presentation.[10,16,28] It is unlikely that all of these symptoms are directly related to the presence of a duodenal adenoma. More likely, duodenal adenomas are coincidental findings in many cases, implicating that a substantial number of adenomas are never discovered. In addition, we found a relatively low number of ampullary adenomas in our series.[8,18,23] This suggests that ampullary adenomas, which are more likely to undergo malignant transformation compared with adenomas originating elsewhere in the duodenum,[8,29] are more frequently missed at diagnostic EGDs. This could be because of the use of front-viewing endoscopes and no side-viewing endoscopes during standard diagnostic EGD. Despite the fact that numerous sporadic duodenal adenomas are probably not detected, prevalence of sporadic duodenal carcinomas in the general population is estimated to be low.[30,31]

The endoscopic interventions performed were standard biopsy, snare polypectomy, and argon plasma coagulation. Complete endoscopic removal was accomplished in a high percentage of cases, most requiring only one treatment session, as has been noted in a recently published study.[14] No complications of endoscopic treatment occurred in our patients. In contrast, surgical treatment resulted in a significant complication rate and even one death. Although we recognize that some duodenal lesions cannot be treated endoscopically and the endoscopic removal rate is therefore subject to selection bias, findings on complications emphasize the preference of endoscopic treatment over surgery. Techniques of endoscopic removal are not standardized.[17] Recent studies propose EMR as option for treatment but point at the relatively high incidence of bleeding as complication.[11,13,15,32] Important advantage of EMR is that the technique permits complete and undamaged removal of the adenoma tissue for histopathological examination, reducing the chance of missing malignant foci because of sampling error and the small size of the standard biopsies taken. Complete endoscopic removal of the adenoma should therefore be the preferred treatment option, particularly in patients without significant comorbidity. Patients who have severe comorbidity at the time of diagnosis do not seem to benefit from treatment, as illustrated by the short survival in the subgroup of patients that underwent no intervention.

We found a recurrence rate of 20% in patient who underwent endoscopic or surgical intervention. This finding is comparable to previously reported rates ranging from 0% to 37%.[12-15] In patients who did not undergo intervention and who were followed endoscopically, no progression or additional adenomas were reported at follow-up. Although adenoma recurrence was considerable, none of the patients in our study cohort developed duodenal cancer during the follow-up period. Consistent with published data of a mean/median age at diagnosis of the sporadic duodenal adenoma ranging from 57 to 69 years,[10-16,18-20,23-25] we found a median of 59 years at the transition of middle to old age. The average time of progression from adenoma to carcinoma is generally expected to be quite long. What management strategy should be followed once complete removal of the sporadic adenoma is ascertained, remains uncertain. Considering the age at diagnosis and long-term outcome after treatment of the duodenal adenoma, the risk of developing a duodenal carcinoma within the range of life expectancy seems to be negligible for the majority of patients. Age and life expectancy at the time of diagnosis should be decisive factors in the choice of follow-up management. Follow-up seems reasonable for patient at younger age (eg, below 60y) without severe comorbidity that limits life expectancy.

In ***Table 3***, to our knowledge, all studies focusing on the association of sporadic duodenal adenomas and colorectal neoplasms are shown.[18-25] Reported prevalence of colorectal neoplasms in patients diagnosed with sporadic duodenal adenomas ranging from 23% to 75%, with colorectal carcinomas diagnosed in 0% to 21% of cases, including the results of the present study with a prevalence of 55% and 7%, respectively. In some patients, screening colonoscopy might have been performed in a regional hospital closer to the patient's place of residence without our knowledge, leading to an underestimation of prevalence of colorectal neoplasms in patients with sporadic duodenal adenomas. In contrast, not all patients with sporadic duodenal adenomas in our series systematically underwent colonoscopy but rather symptomatic patients with an increased a-priori chance of having colorectal lesions, leading to a possible overestimation of the prevalence. In the subgroup of patients who underwent colonoscopy specifically indicated by the diagnosis of the sporadic duodenal adenoma, nearly half were found to have colorectal neoplasms. Although in some of the patients the association could be on the basis of undiagnosed attenuated FAP or MutY homolog (E. coli) gene-associated polyposis,[18,22-24] a shared common pathway between sporadic duodenal and colorectal neoplasm

Table 3. Reports on the association of sporadic duodenal adenomas and colorectal neoplasms.

	Study type	Duodenal adenomas	N	Colonoscopy		CR neoplasia		CR carcinomas		CR adenomas		Adv CR adenoma	
Murray *et al.*[18]	Retrospective case control	Amp & Non-amp	56	34	(61%)	19	(56%)	7	(21%)	12	(35%)	6	(18%)
Ford *et al.*[19]	Retrospective	Amp & Non-amp	35	11	(31%)	4	(36%)	0	(0%)	4	(36%)	3	(28%)
Apel *et al.*[20]	Retrospective	Non-amp	25	22[(1)]	(88%)	16	(73%)	1	(5%)	15	(68%)	ns	-
Schneider *et al.*[21]	Retrospective case control	Amp	26[(2)]	26	(100%)	6	(23%)	2	(8%)	4	(15%)	3	(12%)
Pequin *et al.*[22]	Retrospective case control	Non-amp	44	35	(79%)	13	(37%)	2	(6%)	11	(31%)	8	(23%)
Ramsoekh *et al.*[23]	Retrospective case control	Amp & Non-amp	102	49	(48%)	21[(3)]	(43%)	4	(8%)	17	(35%)	9	(18%)
Lagarde *et al.*[24]	Retrospective case control	Non-amp	29	29	(100%)	18	(62%)	3	(10%)	15	(52%)	4	(14%)
Dariusz *et al.*[25]	Retrospective case control	Non-amp	51	48	(94%)	36	(75%)	5	(10%)	31	(65%)	ns	-
Present study	Retrospective	Amp & Non-amp	54	29	(54%)	16	(55%)	2	(7%)	14	(48%)	7	(27%)

(1) One patient underwent proctoscopy.
(2) Including 7 patients with duodenal cancer.
(3) Two patients had adenomatous lesion on colonoscopy, but no histopathological confirmation was done; these patients were therefore excluded from further analyses.
Advanced adenomas were defined as: size ≥10mm, a villous component, and/or high-grade dysplasia.
Abbreviations: Adv, advanced; Amp, ampullary; CR, colorectal; ns, not specified; Non-amp, non-ampullary.

seems apparent. Therefore, screening colonoscopy in all patients diagnosed with a sporadic duodenal adenoma is warranted, as was previously stated.[17] In patients in whom colonoscopy is too much of a burden, sigmoidoscopy seems a reasonable alternative, as the majority of the associated colorectal lesions were found in the left hemicolon. Computed tomographic colonography can also be considered.[33]

Conclusions and recommendations

Our study illustrates that no consistent approach to the management of sporadic duodenal adenomas was followed. Despite this observation, none of the patients included in our study developed a duodenal carcinoma during the study period, which is of primary concern when a patient is diagnosed with a sporadic duodenal adenoma. We found support for the apparent association between the presence of sporadic duodenal adenomas and colorectal neoplasms. Our recommendation is to aim for complete endoscopic removal of sporadic duodenal adenoma whenever possible. The importance of follow-up after ascertained successful removal largely depends on patient's age and life expectancy. We propose to perform follow-up only in younger patients (eg, under 60y) without relevant comorbidity that limits life expectancy. Given the strong suspicion of an increased risk of colorectal neoplasms, we underline the recommendation to perform colonoscopy in all patients diagnosed with a sporadic duodenal adenoma.[17]

To be able to develop a reliable evidence-based management protocol for patients with sporadic duodenal adenomas, prospective multicenter international studies are considered necessary, as the incidence of sporadic duodenal adenomas is low.

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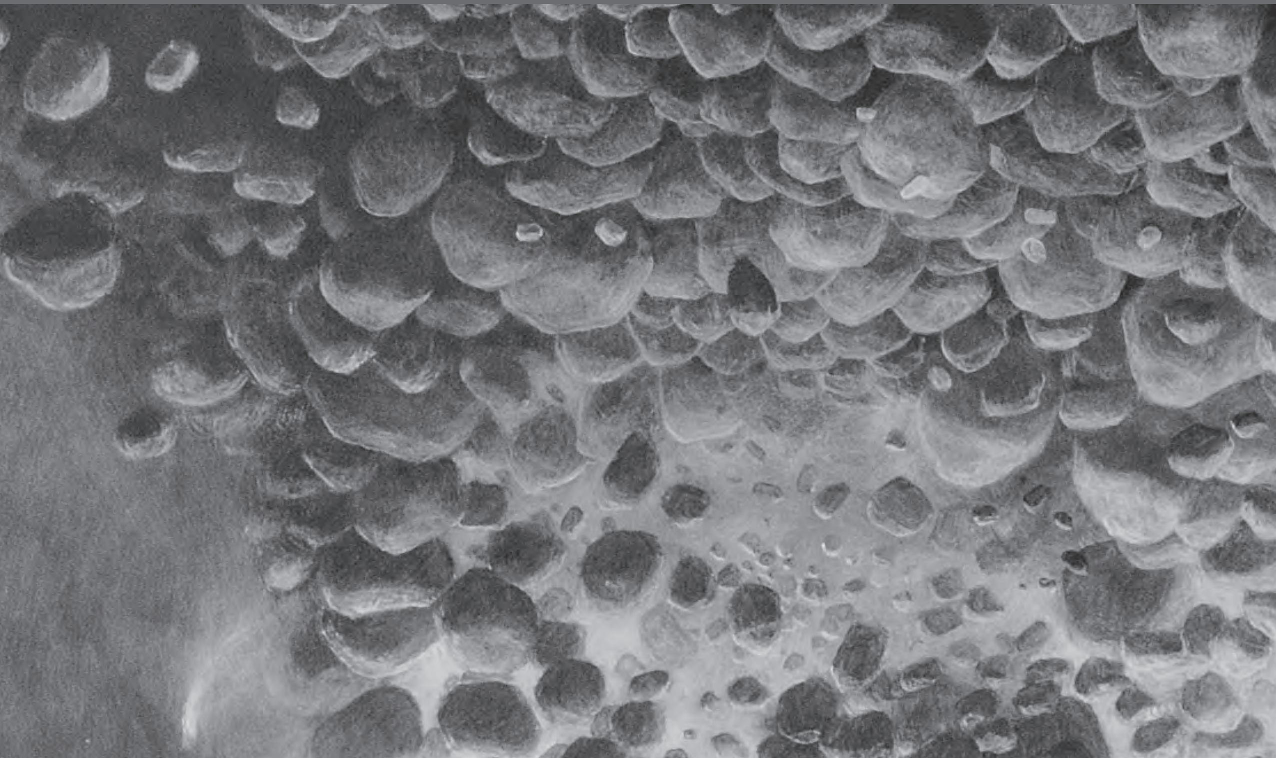

SECTION B

Bridge to clinical evaluation:
tumor cell line studies

CHAPTER 4

The influence of curcumin, quercetin, and eicosapentaenoic acid on the expression of phase II detoxification enzymes in the intestinal cell lines HT-29, Caco-2, HuTu 80, and LT97

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PUBLISHED IN: *NUTRITION AND CANCER*, 2012, 64(6): 856-63.

ABSTRACT

Curcumin, quercetin, and eicosapentaenoic acid (EPA) are three natural compounds with the capacity to reduce adenoma burden in patients with familial adenomatous polyposis (FAP). The mechanistic basis of this anticarcinogenic capacity is largely unknown, but it was suggested that induction of detoxification enzymes is involved. Therefore, the effects of low-dose curcumin, quercetin, and EPA on phase II detoxification enzymes UDP-glucuronosyltransferase (UGT), glutathione S-transferase (GST), as well as on glutathione (GSH) content were analyzed in 4 cell line models of intestinal carcinogenesis. HT-29, HuTu 80, and Caco-2 intestinal cancer cells and LT97 colon adenoma cells from a patient with FAP were treated with low-dose noncytotoxic concentrations of curcumin, quercetin, and EPA. GST enzyme activity was measured by spectrophotometry, and expression of GSTA1, GSTM1, GSTP1, GSTT1, and UGT1 by Western blotting. Cytosolic GSH levels were determined by high performance liquid chromatography. An inducing effect of curcumin and quercetin on GST or UGT was seen in Caco-2, LT97, and HuTu 80 cells. GSH levels were reduced by quercetin and EPA in HT-29 cells and induced by curcumin in Caco-2 cells. In LT97 cells, GST activity and expression was reduced, but UGT1 expression was induced by curcumin and quercetin; whereas EPA only decreased GST or UGT levels. In summary, enhancement of the detoxification capacity by low dose of the potential anticarcinogens curcumin, quercetin, or EPA seems only a minor factor in explaining their anticarcinogenic properties.

Keywords: familial adenomatous polyposis, curcumin, quercetin, eicosapentaenoic acid, chemoprevention, glutathione S-transferase, UDP-glucuronosyltransferase, glutathione, Caco-2, LT97, HuTu 80, HT-29

Abbreviations: APC, adenomatous polyposis coli; COX-2, cyclooxygenase-2; CRC, colorectal cancer; DMEM, Dulbecco's modified Eagle's medium; EPA, eicosapentaenoic acid; FAP, familial adenomatous polyposis; GSH, glutathione; GST, glutathione S-transferase; NSAIDs, non-steroidal anti-inflammatory drugs; ω-3 PUFA, omega-3 polyunsaturated fatty acid; PBS, phosphate buffered saline

Familial adenomatous polyposis (FAP) is a colon cancer predisposition caused by an autosomal dominant germline mutation in the adenomatous polyposis coli (*APC*) gene.[1] The disease is characterized by the development of hundreds to thousands of adenomatous polyps, almost inevitable leading to colorectal cancer (CRC). For this reason, it is recommended to resect the colon to prevent development of CRC.[2] After prophylactic colectomy, however, individuals remain at risk for recurrent rectal or duodenal adenomas and therefore undergo regular endoscopic surveillance.[2] Nonsteroidal anti-inflammatory drugs (NSAIDs) such as sulindac and celecoxib have shown to significantly reduce duodenal or colonic polyp number or size in patients with FAP.[3,4] This pharmacological chemoprevention can be combined with endoscopic surveillance to reduce cancer development or reoccurrence.

The main mechanism of action of NSAIDs in adenoma reduction is assumed to be the inhibition of cyclooxygenase-2 (COX-2), which is overexpressed in 90% of colon carcinomas and in 40% of colon adenomas.[5] Although NSAIDs proved effective, their safety has been a source of controversy. Cardiovascular toxicity of celecoxib was identified in participants of sporadic adenoma trials[6], although there is also evidence of long-term safety of sulindac in a small group of patients with FAP.[7] Of note, the mean age of patients with FAP participating in clinical trials is lower than that of participants in sporadic adenoma trials. It remains unclear whether the use of these drugs is safe in younger individuals. Given the potential adverse effects of NSAIDs, alternative chemopreventive agents are required, which are effective and well tolerated over longer treatment periods.

Curcumin, quercetin, and eicosapentaenoic acid (EPA) are three natural compounds with the capacity to reduce COX-2 expression in the colon cancer cell line HT-29.[8-10] Curcumin is a phenolic compound extracted from the spice turmeric, the powdered rhizome of the plant Curcuma longa. Various pharmacological properties, including the induction of apoptosis and the inhibition of cell proliferation, oxidative stress, and angiogenesis, make curcumin a potential chemopreventive agent.[11] Moreover, its anticancer effect has already been demonstrated for several human cancers and because of its bioavailability in the gastrointestinal tract, curcumin might be particularly suitable to treat gastrointestinal cancers. Clinical trials analyzing the *in vivo* effects of curcumin are still in progress. However, one study showed a reduction in the number and size of polyps in five patients with FAP, treated with 1,440mg curcumin in combination with 60mg quercetin daily for 6 months.[12]

Quercetin is a flavonoid found in many vegetables, fruits, leaves, and grains. Similar to curcumin, quercetin possesses a great potential in the treatment of various diseases due to its wide range of biological effects such as antioxidant and anti-inflammatory capacities.[13] The antitumor effects of quercetin was recently studied in the rodent FAP model $APC^{Min/+}$ mice, where quercetin significantly reduced polyp number and size.[14]

EPA is an omega-3 polyunsaturated fatty acid (ω-3 PUFA) that is found mainly in cold-water fish. The efficacy of EPA as the free fatty acid in the protection of CRC is well supported by animal studies and clinical trials. EPA reduced intestinal adenoma number in the $APC^{Min/+}$ mouse model confirming previous results of the anticarcinogenic activity of ω-3 PUFAs[15-17] and EPA reduced polyp number and diameter in patients with FAP, consuming 2g of EPA daily for

6 months.[18] In addition, the compound was well tolerated. Although the mechanism of action is still unclear, it was suggested that the anticarcinogenic effect of quercetin, curcumin, or EPA might be caused in part by enhancement of detoxification enzymes.[12] Curcumin and/or quercetin have shown to influence the levels of phase II detoxification enzymes glutathione S-transferases (GSTs)[19,20] or UDP glucuronosyltransferases (UGTs) in rats[21], whereas EPA has shown inhibiting[22] and inducing[23] effects on UGTs and GSTs in human HepG2 or modified mouse Hepa-1c1c7 cells, respectively.

UGTs catalyze the reaction of predominantly lipophilic compounds with glucuronic acid[24], whereas GSTs catalyze the conjugation of electrophilic substrates to glutathione.[25] Inducing the activity of these detoxification enzymes could potentially help to protect cells from effect of toxic and (pre)carcinogenic agents, thus reducing the risk of developing (pre) malignancies.[26,27]

Although a reduction in adenoma number and size in patients with FAP has been demonstrated after treatment with curcumin, quercetin, and EPA, the mechanistic basis of this anticarcinogenic effect remains unknown. The aim of the present study is to investigate the effects on phase II detoxification enzymes of low-dose curcumin, quercetin, and EPA that could eventually be reached in clinical studies. The phase II enzymes UGT, and GST/glutathione (GSH) are studied in human (pre)cancerous intestinal cell line models, including an adenoma cell line derived from a patient with FAP (LT97).

MATERIALS AND METHODS

Cell lines

All cell lines, except LT97, were obtained from the American Type Culture Collection (Rockville, MD). HT-29 (clone HTB 38) is a cell line derived from human colorectal adenocarcinoma, HuTu 80 (clone HTB 40) is derived from a human duodenal carcinoma, and Caco-2 (clone HTB 37) is a human colorectal adenocarcinoma cell line. The human LT97 cell line was derived from colorectal microadenomas from a patient with FAP, as described by Richter *et al*.[28]

Cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) high glucose with stable glutamine (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Invitrogen, Paisley, UK), 2% HEPES buffer solution (PAA Laboratories), and 1% minimum essential medium with nonessential amino acids (Invitrogen).

WST-1 cytotoxicity assay

Cytotoxicity of the test compounds was measured as follows: HT-29 and Caco-2 cells were seeded at 2×10^4 cells/well, HuTu 80 at 1.5×10^4 cells/well, and LT97 at 3×10^4 cells/well in 96-well plates. The following day, cells were incubated in complete medium with 0.1% dimethylsulfoxide (DMSO, drug vehicle) containing curcumin (Sigma, St. Louis, MO) in a concentration range of 0.4-400μM, quercetin (Sigma) in a concentration range of 1-1,000μM and eicosapentaenoic acid (EPA, Cayman Chemical Company, MI) in a concentration range of 0.6-600μM. After 24h, cell viability was assessed by adding the cell proliferation reagent

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WST-1 using a 2h incubation time according to the manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany). The absorbance of the samples was measured with a Thermomax microplate reader (Molecular Devices, Wokingham, UK) at wavelength 405nm against a reference wavelength of 620nm. Each measurement of curcumin and quercetin was done in triplicate, and EPA was done in sixplicate. Data shown are mean values derived from 2 or 3 different experiments, respectively. The highest noncytotoxic concentration in each of the 4 cell lines was defined as the highest concentration with maximum cell survival, as deducted from the cell survival curves. These highest noncytotoxic concentrations were used for further experiments.

Cell treatment

2.5×10^6 cells were seeded in $75cm^2$ cell culture flask in complete medium. After 24h, medium containing curcumin, quercetin, or EPA in noncytotoxic concentrations, as determined by WST-1 cytotoxicity assay as described above, was added for 24h. Test compounds were dissolved in DMSO and diluted until the end concentration of DMSO was 0.1%, which was also added to the control cells.

Western Immunoblotting

After incubation for 24h, cells were harvested by scraping, cells were washed thoroughly with phosphate buffered saline (PBS) and centrifuged for 5min at 200×*g*. The cell pellet was taken up in homogenization buffer (0.25M saccharose, 20mM Tris, 1mM dithiothreitol, pH 7.4) and homogenized. Protein concentration of the cell homogenate was determined by the method of Lowry *et al*.[29] Cell homogenates were diluted (2:1) with loading buffer (125mM Tris, 2% SDS, 325mM dithiothreitol, 10% glycerol, 6M urea, and 0.05% bromophenolblue), incubated for 5min at 95°C, and subsequently loaded on 8% or 12% SDS polyacrylamide gel for UGT or GST analysis, respectively.

After electrophoresis, the samples were transferred to nitrocellulose (Whatman GmbH, Dassel, Germany) in a semi-dry blotter (V20-SDB, Scie-Plas, Cambridge, UK). Western blots were blocked with 2% milk powder and incubated with the appropriate primary antibody overnight. As primary antibodies, the mouse monoclonal antibodies WP1, GST class alpha, GST class mu, and GST class pi against UGT1, GSTA1-2, GSTM1- 1, and GSTP1-1 were used, respectively, which were all developed in our laboratory.[30-33] Antibodies against GSTT1-1 were purchased from Dr. E. Juronen (Tartu, Estonia). Polyclonal rabbit antimouse immunoglubulins HRP (Dako Denmark A/S, Glostrup, Denmark) diluted 1:1,000 was used as secondary antibody.

Western blots were independently analyzed by 2 observers with the TotalLab TL100 software (TotalLab, Ltd., Newcastle upon Tyne, UK). Protein bands of untreated cells were set as 100%. Results shown are derived from 5 individual experiments and given in mean ± SD.

GST enzyme activity

GST enzyme activity was measured spectrophotometrically at 340nm according to Habig *et al.*, using 1-chloro-2,4-dinitrobenzene as substrate.[34] Mean values of untreated cells were set as 100%. Results of 5 individual experiments as mean ± SD are shown.

Cytosolic glutathione (GSH) determination

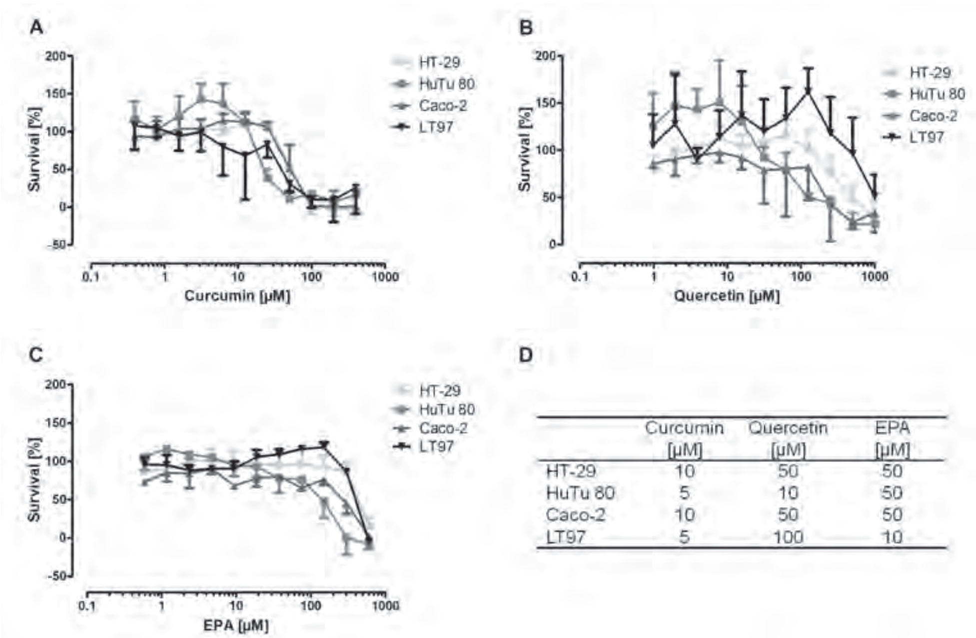
GSH concentrations were analyzed by high-performance liquid chromatography with fluorescent detection. The analysis of GSH was performed as described by Raijmakers *et al.*[35], with minor modifications. Homogenates were diluted in homogenization buffer (0.25M saccharose, 20mM Tris, 1mM dithiothreitol, pH 7.4) and total GSH concentration was determined in 4 experiments. Mean values of untreated cells were set as 100%.

Statistical analysis

The SPSS statistical package (SPSS 16.0 for Windows) was applied for all statistical analyses. Experiments were analyzed by an independent *t*-test. $p<0.05$ was considered statistically significant.

RESULTS

The WST-1 assay was performed to assess the cell viability of the intestinal adenoma and carcinoma cells after treatment with varying concentrations of curcumin (0.4-400µM),



	Curcumin [µM]	Quercetin [µM]	EPA [µM]
HT-29	10	50	50
HuTu 80	5	10	50
Caco-2	10	50	50
LT97	5	100	10

Figure 1. Cytotoxicity of curcumin, quercetin and eicosapentaenoic acid (EPA) in HT-29, HuTu 80, Caco-2, and LT97 cells. A: The cells were exposed to curcumin for 24h at the concentrations 0.4-400µM. Error bars indicate SD of 3 experiments each measured in triplicate. B: Cells were exposed to quercetin at the concentrations 1-1,000µM for 24h. Error bars indicate SD of 3 experiments each measured in triplicate. C: Cells were exposed to EPA for 24h at the concentrations 0.6-600µM. Error bars indicate SD of 2 experiments each measured in sixfold. D: Noncytotoxic concentrations in each of the 4 cell lines, as used in further incubation experiments, are defined as the highest concentration with maximum cell survival deducted from the cell survival curves.

quercetin (1-1,000μM), or EPA (0.6-600μM). The resulting curves show a concentration-dependent inhibitory effect on cell growth for all 3 compounds (***Figure 1A-1C***). From these curves, noncytotoxic concentrations were determined (***Figure 1D***). In subsequent experiments, cells were incubated for 24h using these noncytotoxic concentrations.

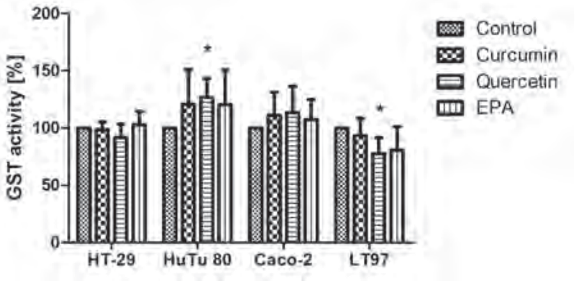


Figure 2. Glutathione S-transferase (GST) enzyme activities of HT-29, HuTu 80, Caco-2, and LT97 cells before and after incubation with curcumin, quercetin, or eicosapentaenoic acid (EPA). Error bars indicate SD of 5 experiments. Enzyme activities of untreated cells are set to 100%. *p<0.05 compared to control.

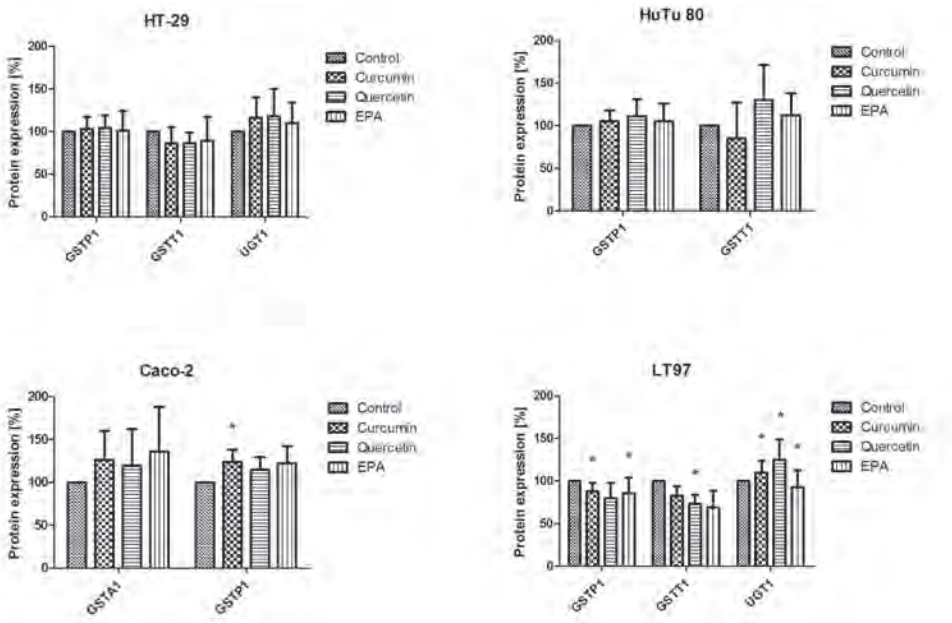


Figure 3. Glutathione S-transferase (GST) and UDP-glucuronosyltransferase 1 (UGT1) protein expression in HT-29, HuTu 80, Caco-2, and LT97 cells before and after incubation with curcumin, quercetin, or eicosapentaenoic acid (EPA). Western blots were quantitatively analyzed. Protein expression of untreated cells are set to 100%. Error bars indicate SD of 5 experiments. A: HT-29 cells express GSTP, GSTT, and UGT1. B: HuTu 80 cells express GSTP and GSTT. C: Caco-2 is the only cell line that expresses GSTA and GSTP. D: LT97 shows the same expression pattern as HT-29 cells. GSTM is not expressed in any of the 4 tested cell lines. *p<0.05 compared to control.

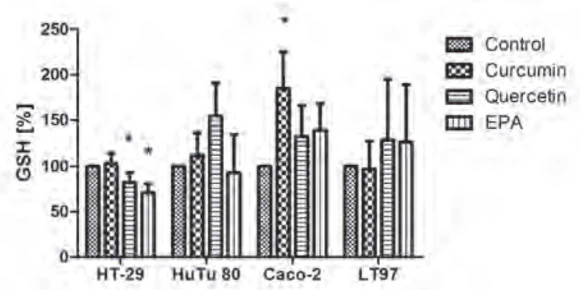


Figure 4. Glutathione (GSH) levels in HT-29, HuTu 80, Caco-2, and LT97 cells before and after incubation with curcumin, quercetin or eicosapentaenoic acid (EPA). Error bars indicate SD of 4 experiments. GSH concentration of untreated cells are set to 100%. *p<0.05 compared to control.

Results of GST enzyme activity measurements are shown in ***Figure 2***. In untreated cells, the highest GST activity is measured in Caco-2 cells with an average activity of 1871±396nmol/min.mg protein, HuTu 80 cells show the lowest activity (264±57nmol/min.mg protein) and HT-29 and LT97 cells showed intermediate GST activity of 537±174 and 455±174nmol/min.mg protein, respectively.

After incubation with the test compounds, no significant change in GST enzyme activity was found in HT-29 and Caco-2 cells. Of all 3 substances investigated, only quercetin significantly induces GST enzyme activity in the HuTu 80 cells, whereas it reduces activity in LT97 cells (both $p<0.05$). To determine which GST classes are affected, Western blot analyses for GSTA1, GSTP1, GSTT1, and GSTM1 were performed (***Figure 3***). GSTM1 is not expressed in any of the cell lines, GSTA is detected only in Caco-2 cells, whereas GSTP1 is detected in all 4 cell lines. In Caco-2 cells, curcumin significantly affected GSTP1 expression. In addition, expression of all UGT1 family enzymes was determined by Western blotting (***Figure 3***). Significant effects on GST and UGT expression were mostly observed in LT97 cells. In these cells, curcumin reduces GSTP1 expression whereas UGT1 expression is induced. Quercetin significantly reduces the expression of GSTT1 and, like curcumin, shows an inducing effect on UGT1 expression. In contrast, EPA decreases both UGT1 and GSTP1 expression.

Results on GSH concentrations are shown in ***Figure 4***. The highest GSH concentration was seen in Caco-2 cells with an average level of 10.1±2.7nmol/mg protein, followed by HT-29 and HuTu 80 cells with average concentrations of 8.2±1.7 and 7.5±2.9nmol/mg protein, respectively. LT97 cells showed a very low cytosolic GSH concentration of 0.26±0.15nmol/mg protein. Quercetin and EPA significantly reduce GSH concentration in HT-29 cells, whereas curcumin significantly induces GSH levels in Caco-2 cells.

DISCUSSION

Curcumin, quercetin, and EPA have been found to reduce cell growth in cancer cell lines[8-10], reduce adenomas/carcinomas numbers in animal models of colon carcinogenesis[14-17], and, most importantly, reduce number and size of colorectal adenomas in patients with FAP[12,18]. In the present study we used four (pre)cancerous intestinal cell line models to analyze the *in vitro* effects of low-dose curcumin, quercetin, and EPA on the detoxification enzymes GST and UGT to investigate whether the protective effects attributed to these three naturally occurring substances have a mechanistic basis in modulating detoxification enzyme processes.

The hypothesis that elevated levels of UGTs, GSTs, and GSH have a protective role in carcinogenesis is firmly established.[24-27] It was shown that low GST activity in the gastrointestinal tract correlates with an increased tumor risk and vice versa.[36] Furthermore, knockout of GSTP in APC^{Min} mice resulted in a sixfold increase in colon adenoma incidence compared to wild type APC^{Min} mice.[37] Transgenic rats containing an extra GSTP gene were shown to be less sensitive to liver carcinogenesis than wild-type rats.[38] On the other hand, recent studies analyzing genetic polymorphisms in *GSTA1*, *GSTM1*, *GSTT1*, *GSTP1*, and *UGT1A1*, which are hypothesized to be related to reduced *in vivo* enzyme activity, have failed to find an association with tumor risk in patients with FAP or sporadic CRC.[39,40] It is noteworthy however, that patient numbers in these studies were low.

Once a (pre)malignancy has developed, GSTP1 is commonly found overexpressed and high levels of GSH have been detected in many human tumors including colon cancer.[24,41] This elevated state, which is present in many different tumor types, might protect cancer cells from chemo- and radiotherapy or oxidative stress-induced apoptosis.[24]

In nonmalignant healthy cells, high levels of GST, UGT, and GSH may be beneficial to protect the cells from oxidative stress and the influence of (pre)carcinogens. Moreover, compounds that increase levels of the phase II enzymes UGT and GST, as well as cytosolic GSH, could act as anticarcinogenic. In the present *in vitro* study only incidental effects of curcumin, quercetin, or EPA on the phase II enzymes were found and effects varied between the cell lines used. For instance, quercetin induced the GST enzyme activity in HuTu 80 cells but reduced it in LT97 cells. Inducing effects were seen only in HuTu 80 and Caco-2 cells. HT-29 cells showed only a lowering effect on GSH, whereas most effects, both enhanced and reduced expression, were seen in the LT97 cells.

Of all three compounds, various effects have been described on colorectal carcinoma cells after at least 6h of incubation at concentrations comparable to those used in the present study.[9,42,43] Effects were found to be strongly dependent on the duration of incubation.[42] The majority of studies on curcumin, quercetin, or EPA in gastrointestinal cell-line models were performed using HT-29 and Caco-2 cells, whereas no studies analyzing GST, UGT, or GSH in HuTu 80 or LT97 cells were previously performed. However, because of differences in concentrations and duration of incubations applied in these studies, comparing the results is not straightforward.

Compared to GST and UGT expression, effects on GSH levels were subject of investigation more often. In our study, Caco-2 cells responded to quercetin by increasing the cytosolic GSH level, supporting the results of a previous study.[44] In HT-29 cells, curcumin was found to significantly increase GSH content after no more than 3h[45], and two studies indicated a raised GSH level after treatment with quercetin at a concentration of 10μM.[44,46] In contrast to curcumin and quercetin, EPA was found to cause a 20% reduction of GSH after 24h of treatment.[47] Our results are in line with these previous findings on curcumin and EPA, but we found quercetin to reduce GSH in HT-29 cells.

Two studies on UGT effects of quercetin in differentiated Caco-2 cells found an induction in experimental settings with 2h and 5wk incubation periods, respectively.[48,49] We did not detect any protein UGT1 expression in Caco-2 cells. However, we used undifferentiated cells in the log phase, which might explain the difference, because cell characteristics do change dramatically after differentiation and their characteristics are known to be diverged significantly in different laboratories.[50,51]

LT97 cells showed no effect on UGT1A1 mRNA levels after 24h treatment with 50μM EPA[52], whereas we found a significant reduction in total UGT1 protein expression after 24h treatment with 10μM EPA. Since we did not discriminate between the various UGT1 family enzymes that might be expressed in LT97 cells, this finding represents a net overall downregulation of UGT1 protein and may obscure differences between the various UGT1 subtypes.

Only three reports exist on GST expression/activity in the cell lines we used. The first reported an induction of GST enzyme activity in HT-29 cells treated with 10-30μM curcumin.[45] In the present study, we did not find any difference in GST enzyme activity after curcumin treatment at 10μM. Secondly, quercetin was found to reduce GSTA1 mRNA expression after 2h of incubation of differentiated Caco-2 cells at concentrations of 25μM[48], whereas in our study 50μM of quercetin showed a nonsignificant increase of GSTA1 protein expression in nondifferentiated cells after 24h incubation period. Lastly, we found a decrease in GSTP1 expression in LT97 cells after incubation with 10μM EPA, whereas in a recent study analyzing the gene expression of LT97 cells under the influence of 50μM EPA, a modulation of the GSTP1 gene was not reported.[52] However, a time-dependent effect of EPA on this colorectal adenoma cell line was clearly present, with more upregulated genes after 24h, as compared to 10h of incubation.

Long term *in vitro* incubation with curcumin in HT-29 cells was performed by Lev-Ari *et al.*[8] and Goel *et al.*[53], resulting in considerable increase of cytotoxicity, when incubating longer than 24h and using concentrations higher than 25μM, which is in agreement with our results on culturing with 10μM curcumin for 24h.

Additional evidence for a time-dependent effect was found in quercetin-treated human leukemic monocyte lymphoma cells.[54] Long-term treatment (12-24h) resulted in decreased levels of GSH leading to an prooxidative and proapoptotic effect, whereas short-term treatment of up to 6h had antioxidative and antiapoptotic effects. Moreover, a rat study indicated effects of curcumin or quercetin as well, with enhanced GST activity in the intestine after 2wk supplementation, whereas glutathione levels were higher in the large intestine only for quercetin.[19] Although the data on long-term treatment are still scarce, one can hypothesize that long-term *in vivo* treatment results in more clear effects on biotransformation systems and leads to increased cytotoxicity, whereas long-term *in vivo* treatment in rats with curcumin or quercetin did reveal no signs of toxicity.[19] However, both *in vivo* and *in vitro* dose-dependent effects are also demonstrated.

These time- and dose-dependent effects might explain the seemingly inconsistent effects on detoxifications enzymes of treatment with fixed doses of curcumin, quercetin, and EPA, at fixed incubation periods in the 4 cell lines investigated here. Moreover, the time- and dose-dependent effects make proper comparison between various studies with different study designs difficult.

In summary, some enhancing effects on detoxification enzymes of low-dose curcumin, quercetin, and EPA are found in the Caco-2 and HuTu 80 cell lines, whereas variable effects were detected in HT-29 and LT97 cells. Overall however, enhancement of the detoxification enzymes does not seem to be an important mechanism explaining the promising results obtained in inhibiting or preventing adenoma formation in patients with FAP.

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CHAPTER 5

Celecoxib and tauro-ursodeoxycholic acid co-treatment inhibits cell growth in familial adenomatous polyposis derived LT97 colon adenoma cells

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Acknowledgments: The authors thank Dr. M. Moret-Hartman for statistical assistance.

PUBLISHED IN: *EXPERIMENTAL CELL RESEARCH*, 2012, 318(7): 819-27.

ABSTRACT

Chemoprevention would be a desirable strategy to avoid duodenectomy in patients with familial adenomatous polyposis (FAP) suffering from duodenal adenomatosis. We investigated the *in vitro* effects on cell proliferation, apoptosis, and COX-2 expression of the potential chemopreventives celecoxib and tauro-ursodeoxycholic acid (UDCA). HT-29 colon cancer cells and LT97 colorectal micro-adenoma cells derived from a patient with FAP, were exposed to low dose celecoxib and UDCA alone or in combination with tauro-cholic acid (CA) and tauro-chenodeoxycholic acid (CDCA), mimicking bile of patients with FAP treated with UDCA. In HT-29 cells, co-treatment with low dose celecoxib and UDCA resulted in a decreased cell growth (14-17%, $p<0.01$). A more pronounced decrease (23-27%, $p<0.01$) was observed in LT97 cells. Cell growth of HT-29 cells exposed to 'artificial bile' enriched with UDCA, was decreased ($p<0.001$), either in the absence or presence of celecoxib. In LT97 cells incubated with 'artificial bile' enriched with UDCA, cell growth was decreased only in the presence of celecoxib ($p<0.05$). No clear evidence was found for involvement of proliferating cell nuclear antigen, caspase-3, or COX-2 in the cellular processes leading to the observed changes in cell growth. In conclusion, co-treatment with low dose celecoxib and UDCA has growth inhibitory effects on colorectal adenoma cells derived from a patient with FAP, and further research on this combination as promising chemopreventive strategy is desired.

Keywords: familial adenomatous polyposis, HT-29, LT97, celecoxib, ursodeoxycholic acid

Abbreviations: *APC*, adenomatous polyposis coli; β2M, beta-2 microglobulin; CA, tauro-cholic acid; CDCA, tauro-chenodeoxycholic acid; COX, cyclooxygenase; DMEM, Dulbecco's modified eagle medium; DMSO, dimethylsulfoxide; FAP, familial adenomatous polyposis; FBS, fetal bovine serum; NSAIDs, non-steroidal anti-inflammatory drugs; PCNA, proliferating cell nuclear antigen; PGE2, prostaglandin E2; qPCR, real-time quantitative polymerase chain reaction; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; UDCA, tauro-ursodeoxycholic acid

Familial adenomatous polyposis (FAP) is characterized by the development of numerous premalignant colorectal adenomatous polyps and caused by a germline mutation in the tumor suppressor adenomatous polyposis coli (*APC*) gene.[1] Prophylactic colectomy, as preventive measurement for the inevitable development of colorectal cancer in these patients, substantially improved prognosis in the past decades.[2] As a result, the mortality pattern has changed with duodenal cancer now being one of the main cancer-related causes of death.[3,4] Lifetime risk of duodenal adenomas approaches 100% in patients with FAP, and approximately 3-4% of patients eventually develop duodenal cancer.[5,6] As duodenal cancer in patients with FAP has been associated with a poor prognosis[7,8], the clinical challenge is to identify high-risk patients with duodenal adenomas and intervene before progression to cancer occurs. To date, prophylactic duodenectomy offers the only chance of a prolonged disease-free interval in patients with FAP with advanced duodenal adenomatosis, but this type of intervention is associated with substantial morbidity and mortality.[9] Chemopreventive treatments would be highly desirable to postpone or even avoid the necessity for radical prophylactic surgery.

In this respect, cyclooxygenase (COX) inhibiting non-steroidal anti-inflammatory drugs (NSAIDs) have been subject to much investigation. Whereas the COX-1 isoenzyme is constitutively expressed in a wide range of tissues and is considered a housekeeping enzyme, the COX-2 isoenzyme is an inducible enzyme that produces prostaglandins in inflammatory and tumorigenic settings.[10] Overexpression of COX-2 is linked to evasion of apoptosis, enhanced cell growth, tumor angiogenesis, and tissue invasion and metastasis through several signalling pathways.[10] Subsequently, studies in which the COX-2 enzyme was targeted by administration of the selective COX-2 inhibitor celecoxib, showed significant decrease in the occurrence of sporadic colorectal adenomas, not only by suppressing the growth of existing adenomas, but also by preventing the formation of new adenomas.[11,12] COX-2 inhibition in a murine model of intestinal polyposis resulted in a substantial decrease in adenoma size and number.[13] Confirming the findings from animal studies, administration of celecoxib was associated with regression of adenomas of both the colon and rectum in patients with FAP.[14] The value of COX inhibiting agents for regression of duodenal polyposis however, is not well established. Sulindac was found to regress small duodenal polyps, but this effect was limited, despite larger effects on colorectal polyposis.[15] Celecoxib was found to significantly reduce duodenal adenomatosis in patients with FAP after 6 months of treatment with high dosage of 400mg twice daily.[16] Unfortunately, clinical trials involving selective COX-2 inhibitors as chemopreventive agents for colorectal cancer have cast doubt on the suitability of these agents for long-term use, due to increased risks of adverse cardiovascular events.[11,12]

A promising strategy is the combination of low dose celecoxib, in order to minimize toxicity, with other substances. A candidate for such a combination regimen is ursodeoxycholic acid. In *in vitro* models of human colonic epithelial cells, ursodeoxycholic acid and taurine-conjugated ursodeoxycholic acid (UDCA) in particular, significantly reduced cytotoxicity of secondary bile acids.[17] Data from clinical studies support the notion of a possible chemopreventive effect of UDCA on development of colorectal neoplasms, in patients with sporadic colorectal adenomas, and patients with ulcerative colitis and primary sclerosing cholangitis.[18-20] UDCA was found to reduce COX-2 expression in a rat model of colon carcinogenesis, suggesting an alternative and possibly complimentary pathway for inhibition of COX-2.[21,22]. Interestingly, a synergistic effect of sulindac and UDCA in the prevention of intestinal adenomas was found in a murine model of FAP.[23]

To explore the chemopreventive potential of low dose celecoxib in combination with UDCA for (duodenal) adenomatosis in patients with FAP, we aimed to investigate the *in vitro* effects on cell growth and COX-2 expression of both substances, in single treatment and in combination. As a model, we used the human epithelial cell line LT97 derived from colorectal micro-adenomas of a patient with FAP. For comparison, we used the well-established HT-29 adenocarcinoma cell line, also derived from human colon. We hypothesize that the growth inhibitory effect of low dose celecoxib is further potentiated by UDCA.

MATERIAL AND METHODS

Cell culture and reagents

The human colon adenocarcinoma cell line HT-29 was obtained from the American Type Culture Collection (Rockville, MD, USA). Additionally, we used the human epithelial cell line LT97, derived from colorectal micro-adenomas of a patient with FAP as previously described.[24] The following reagents were used: celecoxib (99.4% purity) from Kemprotec (Middlesbrough, UK), tauroursodeoxycholic acid (UDCA; >98.0% purity) from TCI Europe (Zwijndrecht, Belgium) and the sodium salts of tauro-cholic acid (CA; ≥97.0% purity) and tauro-chenodeoxycholic acid (CDCA; 98.0% purity) from Sigma-Aldrich (St. Louis, MO, USA). The cells were grown and maintained in 'PC-1 Chemically defined, Serum-free Medium', supplemented with 2mM L-glutamine (Lonza Walkersville, MD, USA) in a humidified incubator at 37°C and 5% CO^2.

Cell survival assays

Cells (15,000 cells/well) were incubated in 96-wells plates (Corning Inc., NY, USA) in 100µl PC-1 medium. After 24h, medium was removed and PC-1 medium containing 0.1% dimethylsulfoxide

Table 1. Incubation conditions used in the experimental design. HT-29 and LT97 cells were treated for 48 and 72 hours with selected concentrations of celecoxib and/or taurine-conjugated bile acids. Selected concentrations were non-lethal dose substracted from cell survival curves (see Figure 1).

		Selected concentrations (µM)			
Condition	Incubation condition	Celecoxib	UDCA	CA	CDCA
1	Control	-	-	-	-
2	Celecoxib	10	-	-	-
3	UDCA	-	1000	-	-
4	Celecoxib + UDCA	10	1000	-	-
5	BA/UDCA50%	-	500	100	100
6	BA/UDCA50%+Celecoxib	10	500	100	100

Abbreviations: UDCA, tauro-ursodeoxycholic acid; CA, tauro-cholic acid; CDCA, tauro-chenodeoxycholic acid; BA, bile acids CA and CDCA.

(DMSO, drug vehicle) and celecoxib in a concentration range of 1.5-100μM, or UDCA, CA, CDCA, and CA/CDCA in a concentration range of 3.1-3200μM were applied. Each concentration was applied in triplicate. After 24h, medium was removed and the cells were incubated with 10% (v/v) WST-1 (Roche Diagnostics, Mannheim, Germany) in PC-1 medium for 2h, after which the absorbance was measured at a wavelength of 450nm, with a background correction read at 620nm, according the manufacturer's instructions. At least four independent experiments were performed.

Based on the resulting cell survival curves, the highest non-cytotoxic concentrations of celecoxib and the conjugated bile acids were selected for the experiments as described below.

Experimental design: incubation conditions

All incubation conditions are shown in ***Table 1***. Three conditions were designed to evaluate the effect of treating cells with celecoxib, UDCA, and their combination at selected concentrations (conditions 2, 3 and 4) for 48 and 72h. Two additional incubation conditions were added to the experimental design. Because gallbladder and duodenal bile in patients with FAP after prophylactic colectomy was found to consist mostly of glycine or taurine conjugated CA and CDCA in equivalent amounts[25], we used a combination of 50% CA and 50% CDCA to mimic the *in vivo* duodenal bile fluid in patients with FAP ('artificial bile'). In all experiments only taurine-conjugated bile acids were used. An earlier intervention study in patients with FAP showed that supplementation with UDCA resulted in duodenal bile that was enriched with UDCA to up to 50% of the total amount of bile acids.[26] To mimic this situation *in vitro*, we exposed cells to UDCA enriched 'artificial bile', with and without celecoxib (conditions 5 and 6). PC-1 medium with 0.1% DMSO (drug vehicle) was used as control condition (condition 1). Cells (4,000 cells/well for cell growth assays, 300,000 cells/well for protein and RNA analysis) were grown for 24h in 96-wells and 6-wells plates (Corning Inc.), respectively. Thereafter, medium was removed and medium with 0.1% DMSO containing celecoxib, UDCA, CA, CDCA and their combinations at selected concentrations as shown in ***Table 1***, was applied for either 48 or 72h. Every 24h, the medium with additives was refreshed. After the 48 and 72h incubation periods, WST-1 assessment was performed as described above. At least six independent incubation experiments were performed. For protein concentration and Western blot analysis, cells were washed with PBS three times and homogenized in 0.25M saccharose/20mM Tris buffer pH 7.4 containing 1mM dithiothreitol (DTT) and 0.1% Triton X-100 and stored at -20°C. At least three independent incubation experiments were performed for protein analyses. For mRNA analysis, cells were harvested in TRIzol (Invitrogen, Carlsbad, CA, USA) and stored at -80°C. Two independent incubation experiments were performed for mRNA analysis.

Measurement of protein concentrations and SDS-PAGE Western blotting

The cell proliferation marker 'proliferating cell nuclear antigen' (PCNA) and the apoptosis marker 'caspase-3' were assessed. Protein concentrations were determined according to the method of Lowry using bovine serum albumin as standard.[27] Equal quantities of protein (10μg and 50μg for PCNA and caspase-3 respectively) were subjected to 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Simultaneously, a molecular weight marker was loaded (Precision Plus Protein Kaleidoscope Standards, Bio-Rad Laboratories, Hercules, CA, USA). After electrophoresis, proteins were transferred (90min at 0.8mA/cm^2) to nitrocellulose transfer membranes (Protan BA 85, Whatman, Dassel, Germany). Membranes were blocked with 2% non-fat dry milk and 0.5% bovine serum albumin in PBS containing 0.05% Tween-20. Immunodetection was performed with the monoclonal mouse anti-PCNA or anticaspase-3 (both from Cell Signaling Technology, Beverly, MA, USA) and the anti-actin antibody (Sigma-Aldrich), the latter as a control to ensure equal protein loading. Detection was performed by incubation with rabbit anti-mouse horseradish peroxidase conjugated antibody (DAKOCytomation, Glostrup, Denmark) and enhanced chemiluminescence by using the GE Healthcare detection system (GE Healthcare, UK). Intensities of the bands were quantified using TotalLab Quant Software (TotalLab Ltd, Newcastle upon Tyne, UK). The mean of the quantification by two independent observers (BvH, HR) was used to calculate overall means per incubation condition.

RNA isolation and real-time quantitative polymerase chain reaction

Total RNA was isolated using TRIzol reagent (Invitrogen) and 1 μg RNA was converted into cDNA according to the instructions provided by the Roche Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics). Detection and quantification of COX-2 messenger RNA was performed via real-time quantitative PCR (qPCR) using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). Analysis of COX-2 expression was performed using the primers 5'-CCGGGTACAATCGCACTTAT-3' and 5'-GGCGCTCAGCCATACAG- 3' (Isogen Life Science, Maarssen, The Netherlands) and SYBR Green (Molecular Probes, Eugene, OR, USA). Specificity of PCR products for COX-2 was confirmed using melting curve analysis and agarose gel electrophoresis. β-2 microglobulin (β2M) was used as a normalizing control (ΔΔCt method). Analysis of β2M was performed with the primers 5'-ATGAGTATGCCTGCCGTGTG- 3' and 5'-CCAAATGCGGCATCTTCAAAC-3' with a specific probe 5'-FAMCGCGTCGTGGGATGGAGACATGTAAGCAGACGCGDabcyl- 3' (Biolegio, Nijmegen, The Netherlands). The β2M product was specified by agarose gel electrophoresis. The PCR procedure was performed in triplicate on each sample from the two independent incubation experiments and the mean of the triplets was used to calculate overall mean of the two experiments.

Statistical analyses

Results are expressed as mean with standard error of mean (SEM). To evaluate differences between the six incubation conditions, the one-way ANOVA-test was performed. When the ANOVA-test was statistically significant (P-value of <0.05, two-sided), statistical significance between different incubation conditions was evaluated using the post hoc Tukey's pairwise comparison. Statistical analyses were performed using GraphPad Prism, version 4.00 (GraphPad Software Inc., San Diego, CA, USA).

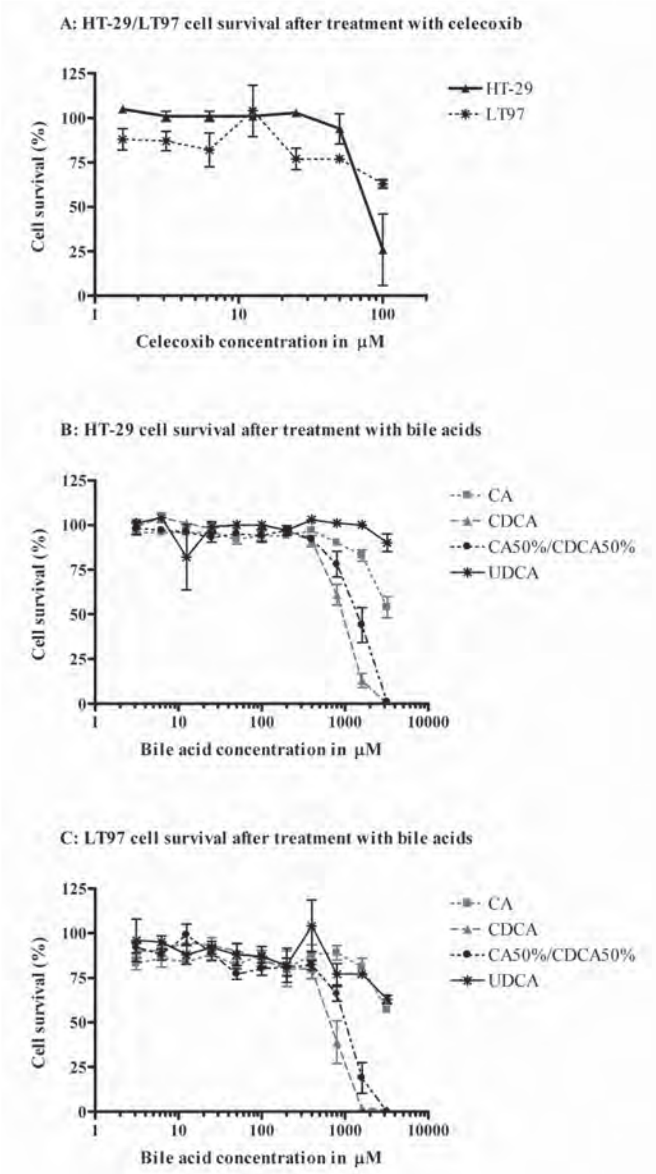

Figure 1. A: Cell survival curves: HT-29 and LT97 cells after treatment with celecoxib. HT-29 cells (B) and LT97 cells (C) after 24h treatment with taurine-conjugated bile acids cholic acid (CA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA) and 'artificial bile' composed of 50% CA and 50% CDCA. Cell survival was measured with WST-1 assay after treatment of the cells at selected concentration ranges of 1.5-100μM for celecoxib and 3.1-3200μM for UDCA, CA, CDCA, or CA/CDCA. The highest non-cytotoxic concentrations of celecoxib and the taurine-conjugated bile acids, represented by the highest concentrations at the plateau of the cell survival curves, were selected for further experiments (for exact concentrations, see Table 1). Note that the X-axis represents a logarithmic scale. *Abbreviations*: CA, tauro-cholic acid; CDCA, tauro-chenodeoxycholic acid; UDCA, tauro-ursodeoxycholic acid.

RESULTS

Cell survival assays

Cell survival curves of HT-29 and LT97 cells after 24h of incubation with celecoxib and the taurine-conjugated bile acids are shown in ***Figure 1***. Based on these curves, we subtracted non-cytotoxic dosages of celecoxib and bile acids for the incubation experiments (see ***Table 1***).

Cell growth assays

Results of the cell growth experiments are shown in ***Figure 2***. In HT-29 cells, incubation with either celecoxib or UDCA alone had no significant effect on cell growth after either 48 or 72h of incubation. However, the combination of celecoxib and UDCA resulted in a decrease in cell growth of 14 and 17% after 48 and 72h, respectively. A more pronounced effect of the

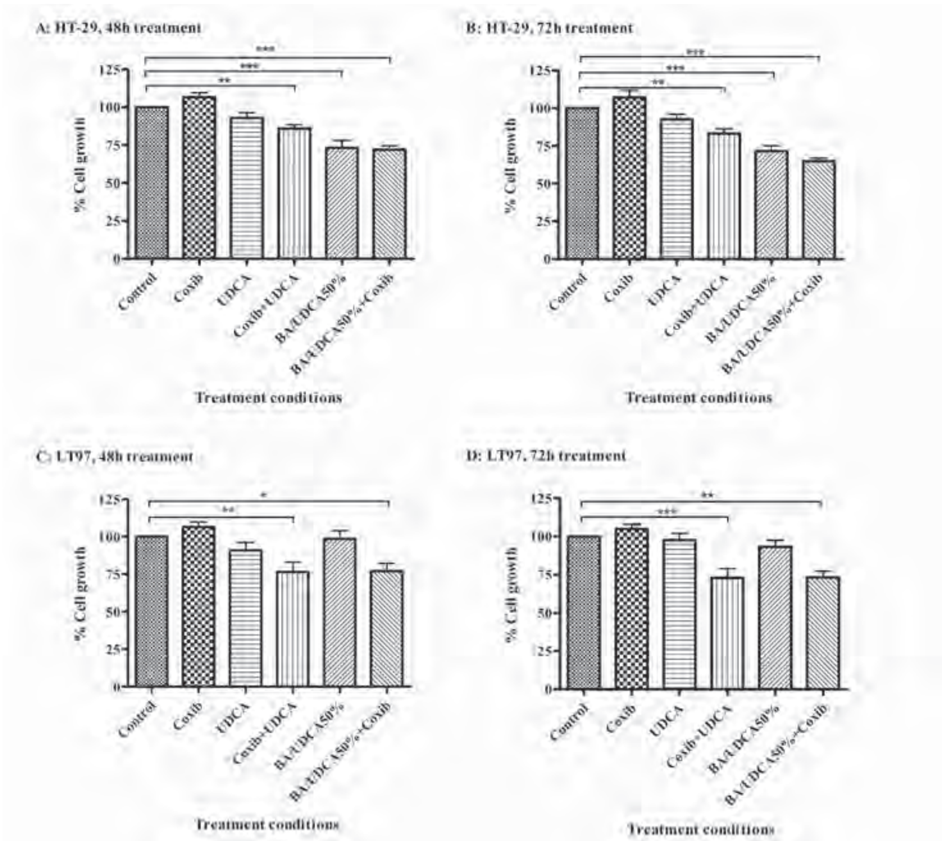

Figure 2. Cell growth of HT-29 and LT97 cells after 48 and 72 hours of treatment with specified substances. Cell viability was assessed by WST-1 assay. Control condition, cells without additives, was taken as reference. One-way ANOVA was statistically significant with $p<0.0001$ in A-D; test results of post hoc tests comparing treatment conditions with control condition: * statistically significant $p<0.05$, ** statistically significant $p<0.01$, *** statistically significant $p<0.001$. *Abbreviations*: Coxib, celecoxib; UDCA, tauro-ursodeoxycholic acid; BA, bile acids tauro-cholic acid and tauro-chenodeoxycholic acid.

combination was observed in LT97 cells, namely 23 and 27% decrease after 48 and 72h of incubation, respectively. When cells were exposed to UDCA enriched 'artificial bile', cell growth decreased significantly in HT-29 cells, either in absence or presence of celecoxib. In LT97 cells incubated with enriched 'artificial bile', a significant decrease in cell growth was found only in the presence of celecoxib. Post hoc comparison of the condition with UDCA enriched 'artificial bile' without celecoxib (condition 5) vs. UDCA enriched 'artificial bile' with celecoxib (condition 6), revealed no differences in cell growth in both cell line models.

Cell proliferation (PCNA) and apoptosis (caspase-3) by Western blot assay

Results of Western blot analyses of the cell proliferation marker PCNA and the apoptosis marker caspase-3 are shown in ***Figures 3*** and ***4***, respectively. No significant changes in levels of PCNA or caspase-3 were found in any of the tested incubation conditions.

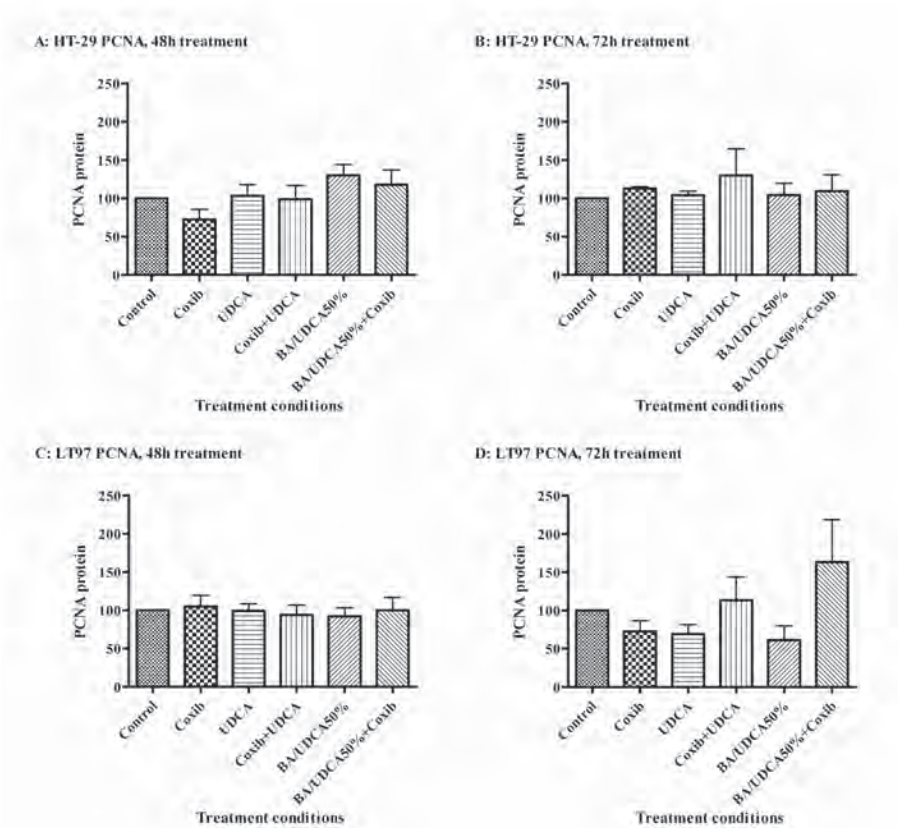

Figure 3. Western blot analyses of PCNA protein in HT-29 and LT97 cells after 48 and 72 hours of treatment with specified substances. Control condition, cells without additives, was taken as reference. One-way ANOVA was not statistically significant with p>0.05 in A-D; post hoc tests were therefore not performed. *Abbreviations*: Coxib, celecoxib; UDCA, tauro-ursodeoxycholic acid; BA, bile acids tauro-cholic acid and tauro-chenodeoxycholic acid.

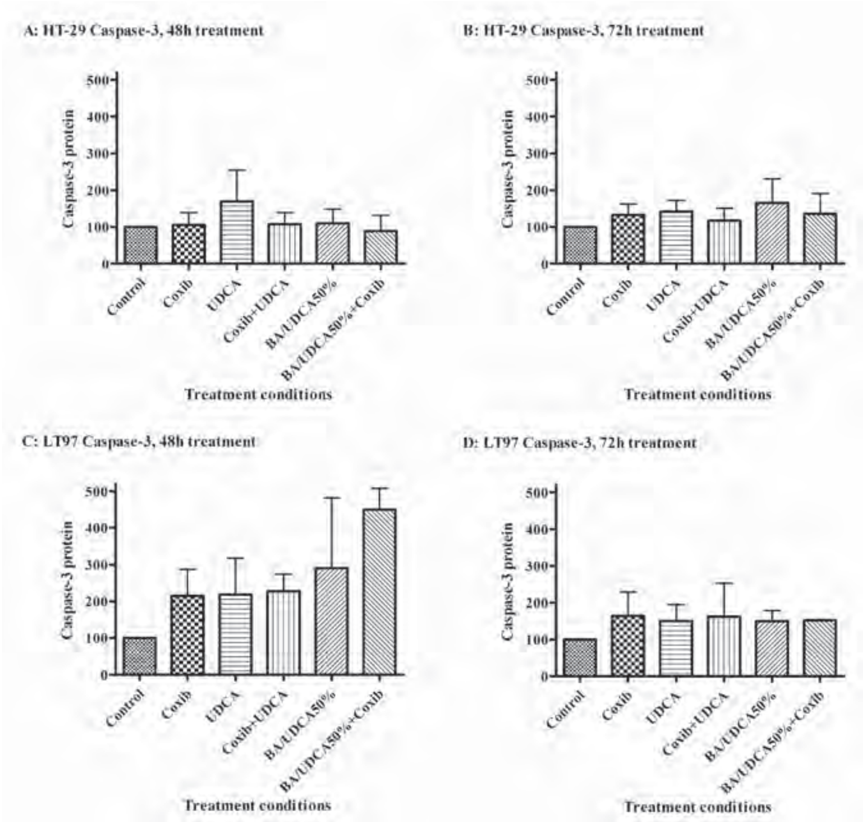

Figure 4. Western blot analyses of caspase-3 protein in HT-29 and LT97 cells after 48 and 72 hours of treatment with specified substances. Control condition, cells without additives, was taken as reference. One-way ANOVA was not statistically significant with p>0.05 in A-D; post hoc tests were therefore not performed; *Abbreviations*: Coxib, celecoxib; UDCA, tauro-ursodeoxycholic acid; BA, bile acids tauro-cholic acid and tauro-chenodeoxycholic acid.

COX-2 expression by qPCR

To determine whether the observed effects on cell growth were associated with down-regulation of COX-2, we assessed COX-2 mRNA expression by qPCR. Results are shown in ***Figure 5***. Note that an increase in mean ΔΔCt values indicates a decrease in COX-2 mRNA expression relative to the control condition. Although effects on mRNA expression seem apparent after 72h of incubation in both HT-29 and LT97 cells, a statistically significant difference was only observed in LT97 cells after 72h of incubation ($p<0.05$). Post hoc tests showed a significant increase in COX-2 mRNA expression after incubation with UDCA enriched 'artificial bile' in the presence of celecoxib ($p<0.05$). Other pairwise comparisons were not statistically significant.

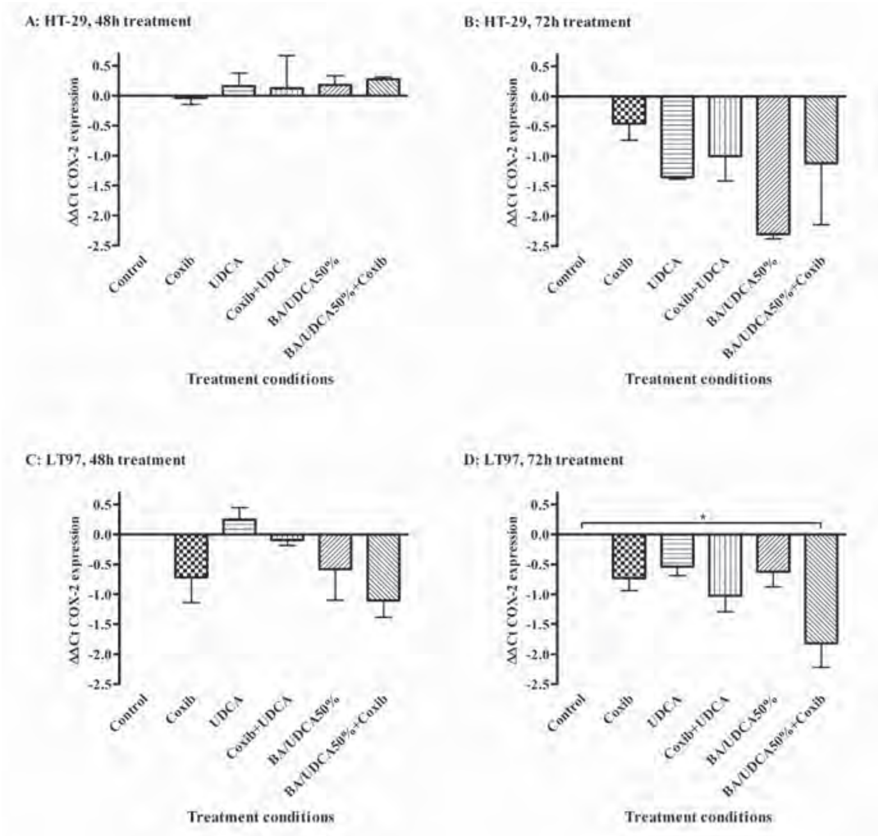

Figure 5. COX-2 mRNA expression in HT-29 and LT97 cells after 48 and 72 hours of treatment with specified substances, assessed by real-time quantative PCR. Control condition, cells without additives, was taken as reference. Results are expressed as value of ΔΔCt. One-way ANOVA was not statistically signifant with p>0.05 in A-C, but statistically significant with p<0.05 in D, post hoc test were therefore only performed in D: * statistically significant with p<0.05; *Abbreviations*: Coxib, celecoxib; UDCA, tauro-ursodeoxycholic acid; BA, bile acids tauro-cholic acid and tauro-chenodeoxycholic acid.

DISCUSSION

The aim of the present study was to explore the chemopreventive potential of celecoxib in combination with taurine-conjugated UDCA with respect to (duodenal) adenomatosis in patients with FAP. Therefore, effects on cell growth and COX-2 expression were investigated in LT97 human colorectal micro-adenoma cells derived from a patient with FAP in comparison with the well-established HT-29 colon adenocarcinoma cells.

We observed that combination treatment with low dose (10μM) of the selective COX-2 inhibitor celecoxib and the tertiary bile acid UDCA (1mM) has a modest growth inhibitory effect on HT-29 cells. This effect was even more pronounced in LT97 colorectal micro-adenoma cells, derived from a patient with FAP. We found no evidence of a growth inhibiting effect of low dose celecoxib (10μM) alone in either HT-29 or LT97 cells, which is in line with results from previous studies in several gastrointestinal tumor cell lines.[28-31] However, considerable antiproliferative effects in HT-29 cells, as well as in other colorectal carcinoma cell lines, were previously described for higher (>25μM) concentrations of celecoxib.[28-30]

Two important differences between the present study and most other studies are to be noted. Firstly, we used serum-free chemically defined PC-1 cell culture medium without fetal bovine serum (FBS). FBS is commonly used in many *in vitro* studies, but the composition varies due to batch-to-batch differences, which may influence results.[32] For example, expression of COX-2 and resistance to apoptosis in HT-29 cells were found to be influenced by culturing in the presence or absence of fetal bovine serum.[33] Secondly, we used low doses of celecoxib (10μM), in contrast to higher doses used in most other *in vitro* studies. As high *in vivo* doses of celecoxib are undesirable because of associated cardiotoxicity[11,12], we applied a near-physiological concentration of celecoxib, close to serum concentrations that were achieved in patients with FAP treated with celecoxib.[34] However, concentrations of celecoxib applied in most *in vitro* cell culture experiments were much higher (>40μM).

After prophylactic colectomy in patients with FAP, the composition of the circulating bile acid pool changes and duodenal bile largely contains glycine- or taurine-conjugated CA and CDCA in nearly equal amounts.[25] In *in vitro* models of human colon cancer cells, non-conjugated UDCA, but more in particular taurine-conjugated UDCA, significantly reduced cytotoxicity of secondary bile acids.[17] By supplementing patients with FAP with high dosage of UDCA, up to 50% enrichment of duodenal bile with UDCA was reached, with a large reduction in concentration of the cytotoxic bile acid CDCA.[26] In addition, a non-significant reduction of COX-2 expression in the duodenal mucosa of these patients was noted immunohistochemically.[26] Because of this reduction in biliary concentrations of CDCA and mucosal COX-2, an inhibition of cell proliferation can be expected after UDCA supplementation. UDCA was also found to have a suppressing effect on COX-2 in a rat model of colon carcinogenesis, suggesting an alternative and possibly complimentary pathway for COX-2-enzyme inhibition.[21,22]

Therefore, we performed our *in vitro* experiments using an adenoma cell line derived from a patient with FAP, and mimicked the *in vivo* situation as studied before[26], in which bile of patients with FAP was enriched with the less cytotoxic UDCA, partly replacing the more cytotoxic CDCA. In addition, we tested whether a synergistic effect of low dose celecoxib and UDCA exists.

In our experiments, cell growth decreased significantly in HT-29 cells exposed to UDCA enriched 'artificial bile', mimicking *in vivo* bile exposure in patients with FAP treated with UDCA, either in absence or presence of celecoxib. In LT97 cells incubated with UDCA enriched 'artificial bile', a significant decrease in cell growth was found only in the presence of celecoxib. These findings suggest that celecoxib exerts additional beneficial growth inhibiting effects in FAP-derived adenoma cells and not in carcinoma cells.

Both LT97 and HT-29 cells were characterized as having mutations in the tumor suppressor gene *APC*.[24,35] Previous studies have demonstrated high levels of COX-2 expression in HT-29 cells[36] and low basal COX-2 expression in LT97 cells[37]. The difference we observed in effects on cell growth between HT-29 and LT97 after incubation with UDCA enriched 'artificial bile' with/without celecoxib, might be explained mainly by the low levels of COX-2 present in LT97 cells, which may be more efficiently inhibited by low dose celecoxib, as compared to HT-29 cells, with high basal levels of COX-2. The relative insensitivity of LT97 cells towards bile acids alone, as noticed in our study, was previously reported.[37] In general, LT97 cells seem to be more resistant to bile acids alone, whereas the combination of low dose celecoxib and bile acids exerts more inhibition of cell proliferation, in comparison to HT-29 cells.

Multiple lines of evidence, including results from *in vitro* studies, animal studies, as well as clinical studies, indicate that inhibition of the increased COX-2 expression, often noticed in carcinogenic processes, at least in part accounts for the anti-proliferative activity of celecoxib.[38] In addition, COX-2 independent pathways have been suggested to be involved in the anti-proliferative effect of celecoxib.[38,39] Anti-proliferative effects with low concentrations of celecoxib, were achieved *in vitro* in COX-2 deficient prostate cancer cells.[40] Furthermore, it was clearly demonstrated that high doses of celecoxib (>50μM) are able to induce apoptosis, but low doses, including the dose we used (10μM), did not induce apoptosis in three colon tumor cells lines, including HT-29.[38] In our study, no significant changes in the levels of PCNA or caspase-3 were found, indicating that the involvement of these pathways in the growth inhibition by celecoxib and UDCA at the concentrations used in our experiments seems modest. We found a significant increase in COX-2 mRNA expression in LT97 cells only after 72h of treatment with UDCA enriched 'artificial bile' in the presence of celecoxib. However, after 72h of incubation with celecoxib, either in the presence of absence of bile acids, in both cell lines a general tendency of increased COX-2 mRNA levels seems to be apparent, which may be explained as resulting from an overshoot mechanism. As a reaction to an initial inhibition of COX-2, transcription may be stimulated by a feedback mechanism, and mRNA levels may be up-regulated, resulting in higher levels after longer incubation periods. The limited effects on COX-2 mRNA levels however, may also further support the concept of involvement of COX-2 independent pathways. Experiments that specifically focus on any of the COX-2 dependent and independent pathways will be required to elucidate the precise mechanism of the growth reduction achieved by combination treatment of low dose celecoxib and UDCA.

In conclusion, *in vitro* incubation with a combination of low dose celecoxib and UDCA exerts growth inhibitory effects in colorectal micro-adenoma cells derived from a patient with FAP, whereas incubation with celecoxib or UDCA alone did not show such an effect. Our *in vitro* results should encourage further research on the low dose celecoxib and UDCA combination therapy as promising chemopreventive strategy for patients with FAP, who have a high-risk of developing gastrointestinal neoplasms.

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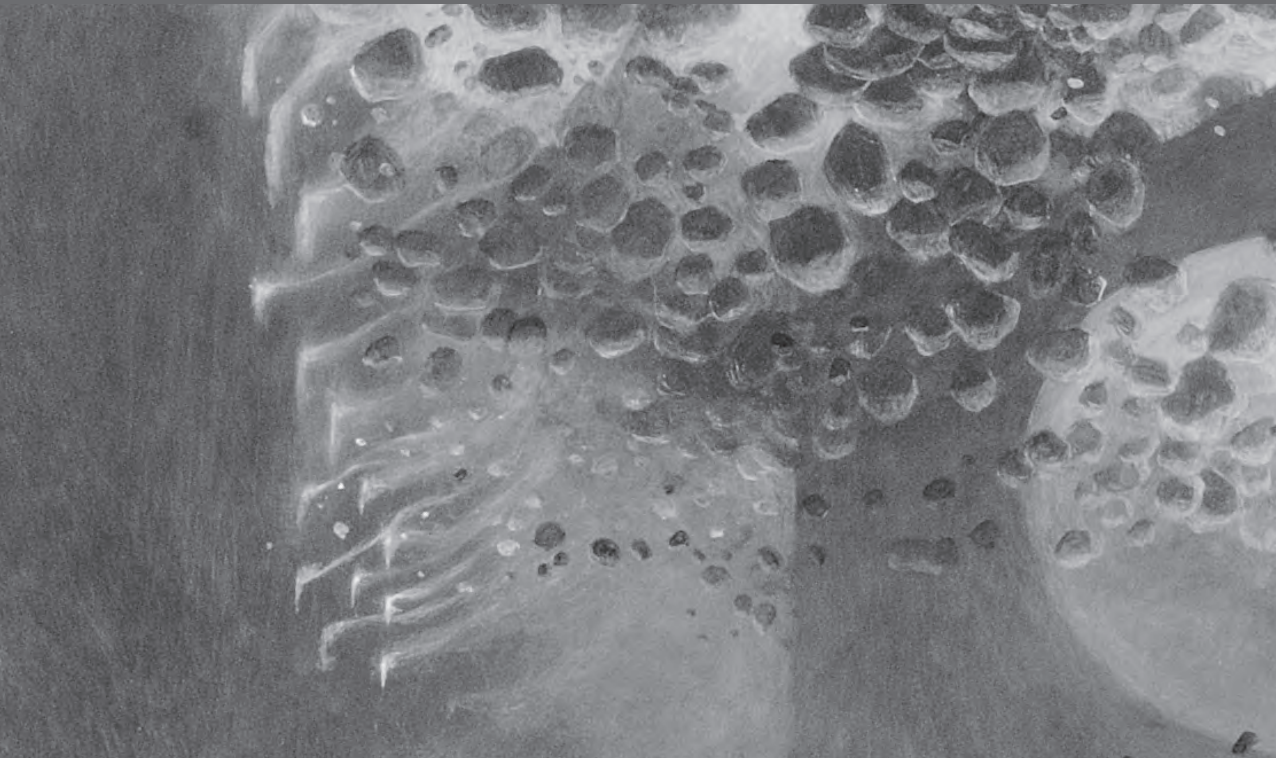
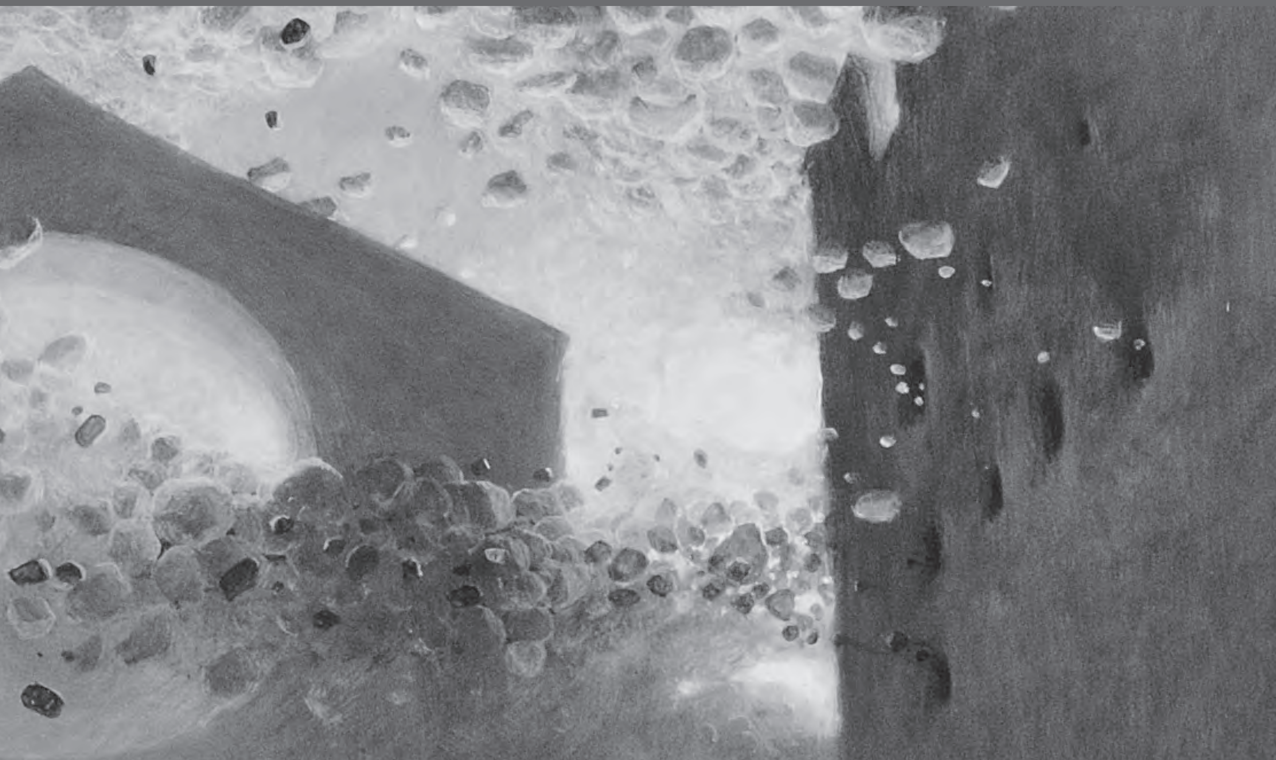
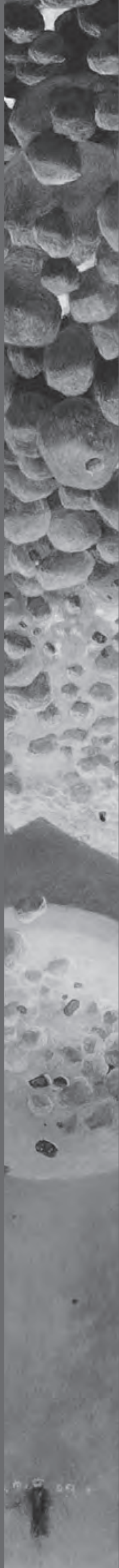


SECTION C

Clinical chemoprevention trial





CHAPTER 6

Ursodeoxycholic acid counteracts celecoxib in reduction of duodenal polyps in patients with familial adenomatous polyposis: A multicentre, randomized controlled trial

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Acknowledgements: The authors thank Dr W Kievit for statistical assistance, MWJ van Vugt-van Pinxteren, CC Cohen, AC de Groot, M de Ley, A Beneken-Kolmer, BM de Klerk, and Dr JW Poley for logistic support, and Drs RPR Adang, LGJB Engels, P Friederich, BE Schenk, NC Talstra, JR Vermeijden, WH de Vos tot Nederveen Cappel, PJ Wahab, BJM Witteman, and FL Wolters for their efforts to refer patients with FAP to our clinic. Dr Falk Pharma (Freiburg, Germany) provided ursodeoxycholic acid and placebo tablets without involvement in study design, collection, analysis and interpretation of data, in the writing of the paper or in the decision to submit it for publication.

PUBLISHED IN: *ORPHANET JOURNAL OF RARE DISEASES*, 2013, 8:118.

ABSTRACT

Due to prophylactic colectomy, mortality in patients with familial adenomatous polyposis (FAP) has changed, with duodenal cancer currently being the main cause of death. Although celecoxib reduces duodenal polyp density in patients with FAP, its long-term use may increase the risk of cardiovascular events and alternatives need to be explored. Preclinical studies suggest that the combination of celecoxib with ursodeoxycholic acid (UDCA) is a potentially effective strategy. We performed a randomized, double-blind, placebo-controlled trial to investigate the effect of celecoxib and UDCA co-treatment on duodenal adenomatosis in patients with FAP. Patients with FAP received celecoxib (400mg twice daily) and UDCA (1000-2000mg daily, 20-30mg/kg/day, n=19) or celecoxib and placebo (n=18) orally for 6 months. Primary outcome was drug efficacy, assessed by comparing pre- and post-intervention duodenal polyp density by blinded review of endoscopic recordings. As secondary outcomes, cell proliferation, apoptosis, and COX-2 levels in normal duodenal mucosa were assessed by immunohistochemistry or real-time quantitative polymerase chain reaction. In intention-to-treat analysis, decreased polyp density was observed after celecoxib/placebo treatment (p=0.029), whereas increased polyp density was observed after celecoxib/UDCA treatment (p=0.014). The difference in change in duodenal polyp density was statistically significant between the groups (p=0.011). No changes in secondary outcomes were observed. Thirty patients (81%) reported one or more adverse events, 16 patients (84%, Common Toxicity Criteria for Adverse Events version 3.0 (CTCAE) grade 1-3) treated with celecoxib/UDCA and 14 patients (78%, CTCAE grade 1-2) treated with celecoxib/placebo. Nine patients (24%) discontinued intervention prematurely, 5 patients (26%) treated with celecoxib/UDCA and 4 patients (22%) treated with celecoxib/placebo. In conclusion, celecoxib reduces duodenal polyp density in patients with FAP, and unexpectedly, high dose UDCA co-treatment counteracts this effect. The benefit of long term use of celecoxib for duodenal cancer prevention needs to be weighed against the (risk of) adverse events.

Keywords: familial adenomatous polyposis; chemoprevention; celecoxib; ursodeoxycholic acid; duodenal adenomatosis; cell proliferation; apoptosis; cyclooxygenase-2

Abbreviations: AEs, adverse events; AMC, Academic Medical Centre Amsterdam; *APC*, adenomatous polyposis coli; β2M, beta-2 microglobulin; CA, cholic acid; CDCA, chenodeoxycholic acid; COX-2, cyclooxygenase-2; CTCAE, Common Toxicity Criteria for Adverse Events; EMC, Erasmus Medical Centre Rotterdam; FAP, familial adenomatous polyposis; H&E, hematoxylin&eosin; LUMC, Leiden University Medical Centre; NSAIDs, non-steroidal anti-inflammatory drugs; PSC: primary sclerosing cholangitis; qPCR, real-time quantitative polymerase chain reaction; RUNMC, Radboud University Nijmegen Medical Centre; UC: ulcerative colitis; UDCA, ursodeoxycholic acid; UMCG, University Medical Centre Groningen

In the past decades, prophylactic colectomy to prevent development of colorectal cancer substantially improved prognosis in patients with familial adenomatous polyposis (FAP).[1] The mortality pattern has changed and duodenal cancer now is the main cancer-related cause of death.[2,3] Lifetime risk of duodenal adenomas approaches 100% [4], and approximately 3-7% of patients develop duodenal cancer.[5,6] As duodenal cancer in patients with FAP has a poor prognosis[7,8], the clinical challenge is to identify patients with high-risk duodenal adenomas and intervene before progression to cancer occurs. Prophylactic duodenectomy may offer a prolonged disease-free interval, but is associated with substantial morbidity and mortality.[9,10] Therefore, chemoprevention would be highly desirable to postpone or even avoid the necessity for radical surgery.

Cyclooxygenase (COX) inhibiting non-steroidal anti-inflammatory drugs (NSAIDs) have been investigated extensively as potential chemopreventive drugs. COX-2 is induced in inflammatory and tumorigenic settings.[11] Overexpression of COX-2, as found in colorectal adenomas and carcinomas, was linked to reduced apoptosis, enhanced cell growth, tumour angiogenesis, tissue invasion, and metastasis.[11] Treatment with the COX-2 inhibitor celecoxib resulted in regression of colorectal adenomas in patients with FAP[12], as well as in significant decrease in sporadic colorectal adenomas.[13,14]

For duodenal polyposis, the value of COX inhibiting agents is not yet established.[15] Sulindac showed regression of small duodenal polyps in patients with FAP[16,17], but had no benefit in controlling periampullary polyposis.[18] The significant reduction in duodenal polyp density after 6 months of treatment with high dose celecoxib in patients with FAP with clinically significant disease was promising.[19]

Unfortunately, suitability of COX-2 inhibitors for long-term use is subject of discussion, due to increased risks of adverse cardiovascular events.[13,14,20] Combining celecoxib with other potentially effective drugs could be a more effective strategy. A candidate drug is ursodeoxycholic acid (UDCA), for a number of reasons. First, the clustering of adenomas around the ampulla of Vater suggests that bile plays a role in duodenal adenomatosis.[21] In *in vitro* models of human colorectal cancer cells, UDCA significantly reduced cytotoxicity of secondary bile acids[22], and celecoxib and UDCA co-treatment inhibited cell growth in colorectal adenoma cells from a patient with FAP.[23] Second, clinical studies showed chemopreventive effects of UDCA on development of colorectal neoplasms, in patients with sporadic colorectal adenomas, and in patients with ulcerative colitis (UC) and primary sclerosing cholangitis (PSC).[24-26] Third, UDCA was found to suppress COX-2 levels in a rat model of colonic carcinogenesis[27], suggesting an alternative pathway for COX-2 inhibition.[28] Finally, in a mouse model of FAP, sulindac and UDCA co-treatment showed synergistic effects in the prevention of intestinal adenomas.[29]

Based on these findings, the aim of the present randomized controlled trial was to examine the effect of celecoxib plus UDCA co-treatment, in comparison to celecoxib plus placebo, on duodenal adenomatosis in patients with FAP. We hypothesized that adding UDCA to the treatment with celecoxib results in a further reduction of duodenal polyp density.

PATIENTS AND METHODS

This clinical trial (http://ClinicalTrials.gov number NCT00808743) was conducted according to ICH Good Clinical Practice and complied with the principles of the amended Declaration of Helsinki and Dutch legislation. Ethical approval was obtained at the initiating centre Radboud University Nijmegen Medical Centre (RUNMC; Protocol approval number 2008/148; CCMO number NL23569.091.08). In the other participating centres, feasibility was approved by the local Medical Ethics Committees. All study participants provided written informed consent. The study was monitored by a RUNMC Safety Monitoring Board.

Study participants

The study population consisted of patients with FAP recruited from the cohort under regular surveillance at the RUNMC, Academic Medical Centre Amsterdam (AMC), Erasmus Medical Centre Rotterdam (EMC), University Medical Centre Groningen (UMCG), and Leiden University Medical Centre (LUMC). The study was conducted between June 2009 and June 2011.

The diagnosis FAP was established either clinically, by the presence of >100 colorectal polyps, or genetically, by the presence of adenomatous polyposis coli (*APC*) gene mutations. Eligible patients were between 18 and 70 years of age, capable of informed consent, had Spigelman stage II or III duodenal adenomatosis at last surveillance duodenoscopy, and had no history of surgical duodenal resection. Exclusion criteria included peptic ulcer disease, inflammatory bowel disease, cardiovascular disease (congestive cardiac failure with New York Heart Association class ≥II; history of ischemic heart disease and/or cerebrovascular disease) or significant cardiovascular risk (at least two of the following risk factors: hypertension, hypercholesterolaemia, diabetes mellitus, ≥2 first degree relatives with cardiovascular event below the age of 55 years), abnormal results on a full blood count or abnormal liver or renal function tests, known intolerability of NSAIDs, sulfonamids, or UDCA, use of NSAIDs or UDCA for >1 week during 6 months prior to study entry, use of lithium, and pregnancy or lactation.

Study procedures

Evaluation at baseline included history taking, physical examination, and clinical laboratory evaluation (full blood count, liver and renal function, cholesterol). Endoscopic procedures were performed using a side-viewing endoscope (Olympus TJF-160, Olympus Medical Systems Europe, Hamburg, Germany) and a forward-viewing endoscope (Olympus GIF-1T-Q160) successively. Endoscopic procedures were recorded digitally. After completion of the recording procedures, six random biopsies of normal appearing mucosa were taken in the second (D2) portion of the duodenum. Two biopsies were fixed in formalin and embedded in paraffin, four biopsies were snap frozen in liquid nitrogen and stored at -80°C. Biopsies were taken using an Olympus Endojaw FB-232U with open forceps diameter 9mm, or a Boston Scientific Radial Jaw 3 with open forceps diameter 8mm (Boston Scientific, Natick, MA, USA). Procedures were repeated after 6 months. At baseline, no biopsies of adenomatous lesions were taken, as this could influence primary outcome.

After completion of pre-intervention duodenoscopy, patients were randomly assigned to one of two treatment groups in an 1:1 ratio. Randomization was performed at the Department of Clinical Pharmacy RUNMC, by a computer-generated schedule, to assign sequentially numbered treatment packs in randomized blocks of four. Patients, physicians, and investigators were blinded to treatment allocation. Patients in group A received orally for 6 months: celecoxib (Celebrex, Pfizer, New York, NY, USA) 400mg twice daily (once daily during the first 2 weeks), in combination with UDCA (Ursofalk, Dr Falk Pharma, Freiburg, Germany). Patients in study group B received orally for 6 months: celecoxib 400mg twice daily (once daily during the first 2 weeks), in combination with an UDCA identical-appearing placebo (Dr Falk Pharma). UDCA/placebo was given in two daily doses, with total daily UDCA dose based on body weight: ≤50kg: 1000mg, 50-70kg: 1500mg, >70kg: 2000mg (20-30mg/kg/day). UDCA starting dose was 500mg, which was raised with 500mg every 2 weeks until maximum dose was reached. The placebo contained lactose and cellulose.

Information on adverse events (AEs) was obtained during patient contacts by telephone at 1 and 3 months, and prior to post-intervention duodenoscopy at 6 months. Monitoring of blood pressure and clinical laboratory parameters was performed at 1 and 6 months. AEs were graded as defined by the Common Toxicity Criteria for Adverse Events version 3.0 (CTCAE v3.0).[30] Compliance was monitored by means of pill counts and review of diaries completed by the patients.

Disclosure of randomization was performed by the Department of Clinical Pharmacy RUNMC on December 10th 2012, after completion of assessment of recorded duodenoscopies and all tissue analyses.

Assessment of recorded endoscopic procedures

Endoscopic recordings were analyzed using qualitative assessment of duodenal polyp density, as previously described in patients with FAP for the colorectum[12] and duodenum[19]. In short, five gastroenterologists experienced in management of FAP (ED, JD, JJK, AMJL, FMN), independently scored the blinded pairs of pre- and post-intervention videos of each patient, shown in random order. Pairs were scored as no change (scored as 0), clinical improvement (scored as +1), or clinical deterioration (scored as -1) in polyp density. Based on the scores of the five gastroenterologists, mean scores of change in duodenal polyp density were calculated for each patient. Patients that discontinued intervention prematurely were included in intention-to-treat analysis with a score of change in duodenal polyp density of -0.5.

Immunohistochemical staining for cell proliferation, apoptosis, and COX-2

Tissue sections of 4µm were cut from paraffin blocks, mounted on electrostatic slides (Super Frost Plus, Menzel-Gläser, Baunschweig, Germany) and stained with Hematoxylin & Eosin (H&E). Only samples with normal histology (non-dysplastic and non-adenomatous mucosa), as verified by an expert pathologist (IDN), were used for further analyses.

Tissue sections were deparaffinized and dehydrated. Endogenous peroxidase was blocked with 3% hydrogen peroxide. Subsequently, heat-induced antigen retrieval was performed in sodium citrate buffer (10mmol/L, pH=6). Cell proliferation activity was assessed after staining for 1 hour at room temperature with mouse anti-human MIB-1 monoclonal antibody (Dako A/S, Glostrup, Denmark) at dilution 1:200. MIB-1 recognizes the Ki-67 nuclear antigen of dividing cells.[31] Apoptosis was assessed by staining overnight at 4°C with mouse anti-human M30 CytoDEATH monoclonal antibody (Roche Diagnostics, Mannheim, Germany) at dilution 1:400. M30 recognizes cleaved cytokeratin 18, expressed in epithelial cells during early apoptosis.[32] COX-2 was assessed by staining overnight at 4°C with mouse anti-human COX-2 monoclonal antibody (Cayman Chemical, Ann Arbor, MI, USA) at dilution 1:100. Visualization of MIB-1 was achieved using the Brightvision (1:1)/BrightDab detection system (Immunologic, Duiven, The Netherlands), whereas M30 and COX-2 were visualized using the avidin-biotin peroxidase complex method (Vector Laboratories, Burlingame, CA, USA). Mayer hematoxylin counterstaining was applied. Tissue sections of colorectal carcinomas were used as positive controls.

Evaluation of immunohistochemical staining and scoring

Tissue samples were independently evaluated by light microscopy (Leica Microsystems, Rijswijk, The Netherlands) by two investigators (BWHvH, MEV-B). If scores differed, a consensus agreement was reached during re-evaluation. A random selection of 10% of scores were re-evaluated and verified by an expert pathologist (IDN). Cell proliferation index was expressed as percentage of MIB-1 positive epithelial cells in areas of the tissue section with well-orientated crypt-villi architecture. Apoptotic index was expressed as number of M30 positive epithelial cells per mm^2 tissue area. COX-2 staining in epithelial cells was scored as previously described[33]: 0, no staining; 1, weak cytoplasmatic and membranous staining (may contain strong staining in <10% of cells); 2, moderate-to-strong staining in 10-90% of cells; and 3, strong staining in >90% of cells.

RNA isolation and real-time quantitative polymerase chain reaction (qPCR) for COX-2

One biopsy sample of each location was weighed and taken up in 200µl TRIzol (Life Technologies, Pailey, UK). Tissue was homogenized by 10 strokes with a Teflon pestle. After homogenization, another 600µl TRIzol was added. Total RNA was extracted according to the manufacturer's instructions (Life Technologies) with a slight modification: prior to precipitating the RNA with isopropyl alcohol, 7.5µg RNAse-free glycogen was added to the aqueous phase. Approximately 1 µg RNA was converted into cDNA according to the instructions provided by the Roche Transcriptor High Fidelity cDNA synthesis kit (Roche Diagnostics). Detection and quantification of COX-2 messenger RNA was performed by qPCR using the CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Analysis of COX-2 expression was performed using two different COX-2 specific primer sets: forward 5'-GGCGCTCAGCCATACAG-3'(exon 1) with reverse 5'-CCGGGTACAACTGCACTTAT-3'(exon 2) and forward 5'-GGCGCTCAGCCATACAG-3'(exon 1) with reverse 5'-TCTTGTCAAAAATTCCGGTG-3' (exons 2 and 3) (Isogen Life Science, Maarssen, The Netherlands). PCR products were detected with SYBR Green (Molecular Probes, Eugene, OR, USA). Specificity of COX-2 PCR products was checked using melting curve analysis and agarose gel electrophoresis. Levels of β-2 microglobulin (β2M) mRNA were used as a normalizing control. Analysis of β2M was performed with the primers 5'-ATGAGTATGCCTGCCGTGTG- 3' and 5'-CCAAATGCGGCATCTTCAAAC-3' with a specific probe 5'-FAMCGCGTCGTGGGATGGAGACATGTAAGCAGACGCGDabcyl- 3' (Biolegio, Nijmegen, The Netherlands). The β2M product was checked by agarose gel electrophoresis. PCR procedures were performed in triplicate or quadruplicate and mean Ct values were calculated.

Statistical analysis

Baseline characteristics were expressed as percentage or medians with range when appropriate. Continuous variables were considered to be not normally distributed. Differences between treatment groups on continuous variables were tested using Mann-Whitney U test, and differences on discrete variables were examined using Chi-square test, or Fisher's exact test when appropriate. Differences on continuous and ordinal variables within treatment groups, comparing pre- and post-intervention measurements, were examined using Wilcoxon Signed Rank test and McNemar's test, respectively. Analyses of primary outcome were performed on an intention-to-treat basis, with a per-protocol analysis as sensitivity analysis. Based on an assumed relevant and detectable reduction of 20% in polyp size as primary outcome, sample size was set at 40 patients per treatment group to demonstrate a significant difference at the 5% significance level with a statistical power of 80%. A p-value of <0.05 (2-sided) was considered statistically significant. Statistical analysis was performed using SPSS statistical software version 21 (IBM SPSS, Chicago, IL, USA).

RESULTS

Patient characteristics

The CONSORT diagram of the study is depicted in ***Figure 1***. Of all patients with FAP that were under regular surveillance in the five participating University hospitals, 94 patients were eligible for inclusion. Twenty-three patients were excluded based on exclusion criteria and 29 patients declined informed constent. Forty-two patients underwent initial duodenoscopy, of which five patients were not randomized: four had insufficient polyps and one patient required treatment because of advanced duodenal adenomatosis. Thirty-seven patients were randomized: 19 patients received celecoxib & UDCA (group A) and 18 patients received celecoxib & placebo (group B). Patient characteristics are depicted in ***Table 1***. Due to technical failure, either pre- or post-intervention recordings could not be analysed in five patients. Consequently, thirty-two patients (group A, n=17; group B, n=15) were analysed on an intention-to-treat basis for the primary outcome. Nine patients (24.3%) discontinued intervention prior to duodenoscopy at six months. Consequently, per-protocol analysis was performed on 23 patients (group A, n=12; group B, n=11).

Primary Outcome: change in duodenal polyp density

In the intention-to-treat analysis, clinical deterioration (n=17, median= -0.2, range:-0.6-+0.4) in duodenal polyp density was observed in group A (Wilcoxon Signed Rank, p=0.014), receiving celecoxib & UDCA, while clinical improvement (n=15, median=0.6, range:-0.5-+1.0)

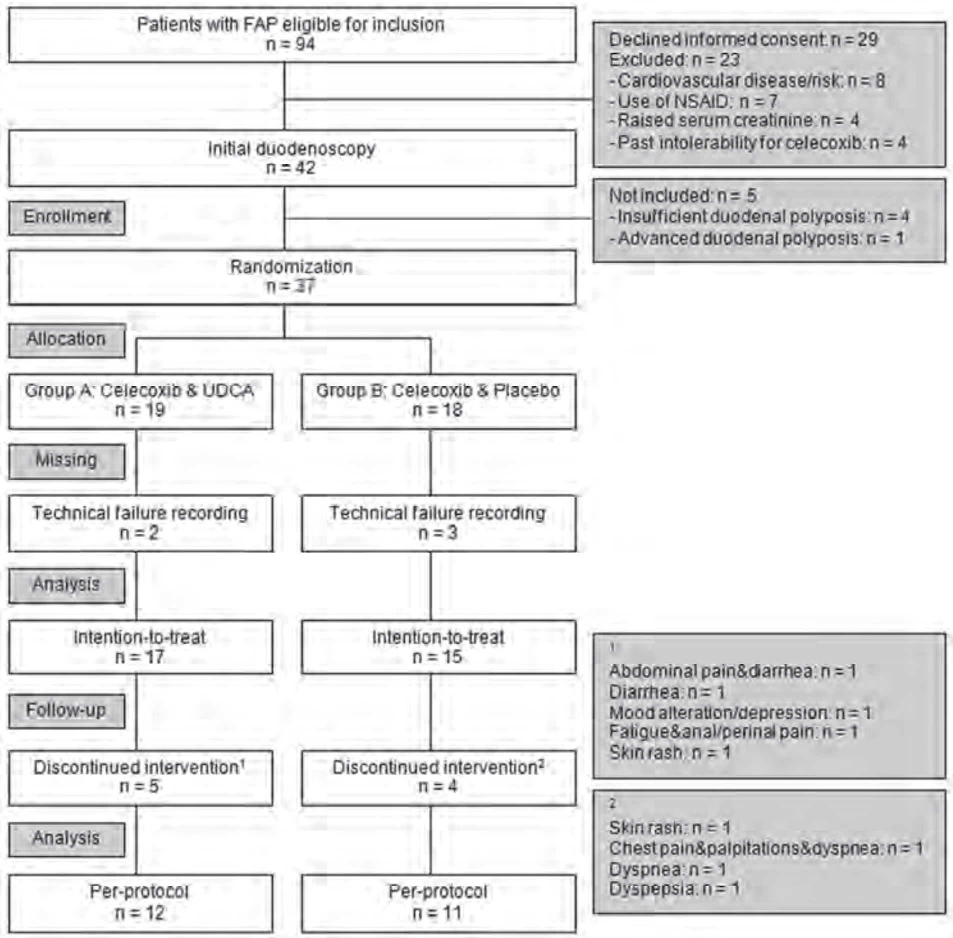

Figure 1. CONSORT diagram. *Abbreviations*: FAP, familial adenomatous polyposis; NSAID, non-steroidal anti-inflammatory drugs; UDCA, ursodeoxycholic acid.

was observed in group B (Wilcoxon Signed Rank, p=0.029), receiving celecoxib & placebo (***Figure 2***). The difference in mean score of change in duodenal polyp density was statistically significant between groups (Mann-Whitney U, p=0.011).

In the per-protocol analyses, the difference in mean score of change in duodenal polyp density between group A (n=12, median=-0.2, range:-0.6-+0.4) and group B (n=11, median=0.8, range:0.0-+1.0) was more pronounced (Mann-Whitney U, p<0.001). Clinical deterioration in duodenal polyp density observed in group A was not statistically significant (Wilcoxon Signed Rank, p=0.271), in contrast to the clinical improvement observed in group B (p=0.004).

Table 1. Base-line characteristics of patients with FAP.

	Overall population	Group A: Celecoxib&UDCA	Group B: Celecoxib&Placebo	p-value
Number of patients	37	19	18	
Age at study entry, median/range (yr)	42/22-67	42/22-67	41/27-64	0.964(3)
Sex (n, %)				0.618(4)
Male	18 (48.6)	10 (52.6)	8 (44.4)	
Female	19 (51.4)	9 (47.4)	10 (55.6)	
Participants per centre (n, %)				0.932(5)
RUNMC	18 (48.6)	10 (52.6)	8 (44.4)	
AMC	10 (27.0)	4 (21.1)	6 (33.3)	
EMC	4 (10.8)	2 (10.5)	2 (11.1)	
UMCG	3 (8.1)	2 (10.5)	1 (5.6)	
LUMC	2 (5.4)	1 (5.3)	1 (5.6)	
Body Mass Index, median/range (kg/m^2)	25.6/18.8-34.5	26.0/19.2-34.5	25.6/18.8-33.1	0.408(3)
Diagnosis FAP				0.660(5)
Clinical only	6 (16.2)	4 (21.2)	2 (11.1)	
APC gene mutation	31 (83.8)	15 (78.9)	16 (88.9)	
Age at primary CR surgery, median/range (yr)	21/7-60	22/7-60	18.5/11-48	0.298(3)
Time since primary CR surgery, median/range (yr)	18/1-38	17/1-33	20.5/8-38	0.178(3)
Type of primary CR surgery				0.738(5)
IRA	18 (48.6)(1)	10 (52.6)(1)	8 (44.4)	
IPAA	14 (37.8)	6 (31.6)	8 (44.4)	
Ileostomy	5 (13.5)	3 (15.8)	2 (11.1)	
Secondary CR surgery (n, %)	11 (29.7)	5 (26.3)	6 (33.3)	0.641(4)
Spigelman stage at last surveillance before entry				0.985(4)
II	19 (51.4)(2)	10 (52.6)	9 (50)(2)	
III	17 (45.9)(2)	9 (47.4)	8 (44.4)(2)	

(1) Including one patient who underwent ileosigmoid anastomosis; (2) In 1 case exact data on last previous surveillance duodenoscopy was missing; (3) The p-value was calculated using the Mann-Whitney U test; (4) The p-value was calculated using the chi-square test; (5) The p-value was calculated using the Fisher's exact test

Abbreviations: FAP, familial adenomatous polyposis; APC, adenomatous polyposis coli; CR, colorectal; IRA, Ileorectal anastomosis; IPAA, Ileal pouch-anal anastomosis; Ileostomy, proctocolectomy with ileostomy; UDCA, ursodeoxycholic acid; AMC, Academic Medical Centre Amsterdam; EMC, Erasmus Medical Centre Rotterdam; LUMC, Leiden University Medical Centre; RUNMC, Radboud University Nijmegen Medical Centre; UMCG, University Medical Centre Groningen

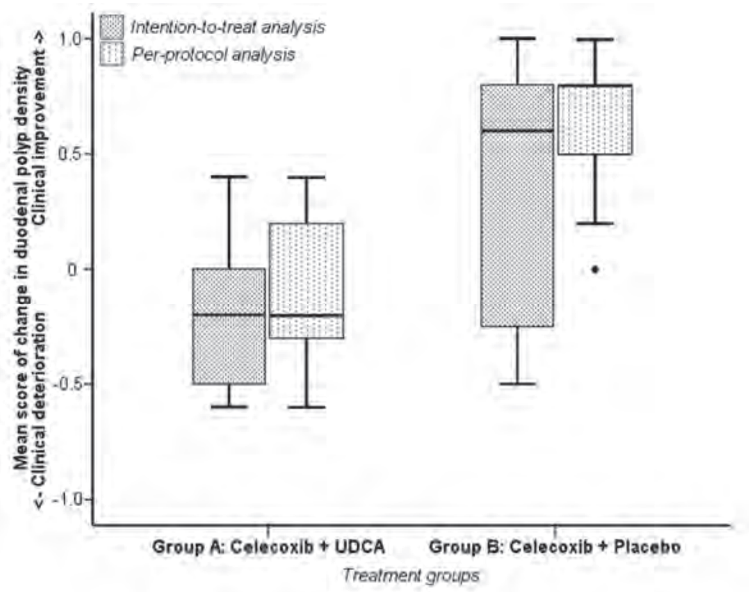

Figure 2. Box-Whisker plots of intention-to-treat and per-protocol analysis. Intention-to-treat analysis of mean score of change in duodenal polyp density comparing duodenoscopic recordings pre- and post-intervention with either celecoxib & UDCA (group A) or celecoxib & placebo (group B): clinical deterioration in group A (n=17, Wilcoxon Signed Rank, p=0.014), clinical improvement in group B (n=15, Wilcoxon Signed Rank, p=0.029); difference in mean score between groups statistically significant (Mann-Whitney U, p=0.011). Per-protocol analysis: clinical deterioration in group A (n=12, Wilcoxon Signed Rank, p=0.271), clinical improvement in group B (n=11, Wilcoxon Signed Rank, p=0.004); difference in mean score between groups statistically significant (Mann-Whitney U, p<0.001); *Abbreviation*: UDCA, ursodeoxycholic acid.

Secundary Outcome: cell proliferation, apoptosis, and COX-2 immunohistochemistry

Changes in cell proliferation, apoptosis, and COX-2 were evaluated in all patients that completed the intervention period and underwent pre- and post-intervention duodenoscopy, with one additional patient excluded in group B of whom post-intervention biopsies could not be assessed (n=27).

Median difference in cell proliferation pre- versus post-intervention was not statistically significant between both treatment groups (group A: n=14, median difference =-5.0%, range=-20.0%-10.0%; group B: n=13, median difference=0.0%, range:-15.0%-20.0%; Mann-Whitney U, p=0.141). The median decrease in cell proliferation of 5.0% observed in group A was not statistically significant (Wilcoxon Signed Rank, p=0.057).

No M30 positive apoptotic epithelial cells were scored in any of the evaluated samples, except for positive control samples.

COX-2 staining was scored as either moderate-to-strong or strong staining in all evaluated samples. No difference in COX-2 staining was seen comparing pre- and post-intervention: in group A, a decreased score was observed in 2 patients, an equal score in 8 patients, and an increased score in 3 patients (McNemar, p=1.000). In group B, a decreased score was observed in 4 patients, an equal score in 6 patients, and an increased score in 2 patients (McNemar, p=0.688).

Secondary Outcome: COX-2 mRNA analyses

COX-2 mRNA expression was also evaluated in all patients that completed the intervention period, with the noted one additional patient excluded in group B (n=27).

In 12 patients (group A: n=6, group B: n=6), no measurable COX-2 mRNA levels were present in either pre- or post-intervention sample. In all other cases, low COX-2 mRNA levels seemed present, but specificity of PCR products could not be confirmed by melting curve analyses and agarose gel electrophoresis. Experiments in which the second set of specific COX-2 primers were used, showed the same results. Simultaneous qPCR analyses on colorectal cancer tissue samples showed high levels of COX-2 specific qPCR products.

Adverse Events and Compliance

AEs were analysed for all randomized patients (n=37). An overview of all 58 AEs reported by 30 patients (81.1%) is shown in ***Table 2***. In group A (n=19), 10 grade 1, 18 grade 2, and 6 grade 3 AEs were reported by 16 patients (84.2%), whereas in group B (n=18), 9 grade 1, and 15 grade 2 AEs were reported by 14 patients (77.8%) (Fisher's exact, p=0.114). Five patients (26.3%) discontinued intervention in group A, due to complaints of abdominal pain and diarrhea (n=1), diarrhea (n=1), mood alteration/depression (n=1), fatigue and anal/perianal pain (n=1), and skin rash (n=1). Four patients (22.2%) discontinued intervention in group B, due to complaints of skin rash (n=1), chest pain, palpitations, and dyspnea (n=1), dyspnea (n=1), and dyspepsia (n=1) (Fisher's exact, p=1.000). Two patients in group A reported insomnia and edema of the lower limbs respectively, which resolved after reducing the celecoxib dose to halve the standard trial dose. Both patients completed the intervention period and were included in the analyses.

Compliance was evaluated in all patients that completed the 6 months intervention period (n=28). In group A, the median compliance for celecoxib and UDCA was 98.4% (range: 82.4-100%) and 96.8% (range: 42.2-100%), respectively. In group B, the median compliance for celecoxib and placebo was 99.4% (range: 79.1-100%) and 97.0% (range: 80.3-100%), respectively.

Table 2. Adverse Events in patients with FAP treated with either celecoxib & ursodeoxycholic acid or celecoxib & placebo.

		Treatment groups	
CTCAE Category	**Name adverse event**	**Group A n=19**	**Group B n=18**
Auditory/Ear	Otitis, middle ear	1 (0/1/0)	0
Blood/Bone marrow	Anemia - hemoglobin	1 (1/0/0)	0
	Leukopenia	1 (0/1/0)	0
Cardiac arrhythmia	Palpitations	0	2 (2/0/0)
Constitutional symptoms	Fatique	2 (0/1/1)	1 (0/1/0)
	Insomnia	1 (0/0/1)	0

Table 2. Adverse Events in patients with FAP treated with either celecoxib & ursodeoxycholic acid or celecoxib & placebo. *(Continued)*.

CTCAE Category	Name adverse event	Treatment groups Group A n=19	Group B n=18
Dermatology/Skin	Hair loss - scalp	1 (1/0/0)	0
	Rash	1 (0/1/0)	1 (0/1/0)
Gastrointestinal	Constipation	2 (2/0/0)	2 (2/0/0)
	Diarrhea	2 (1/1/0)	2 (2/0/0)
	Heartburn/dyspepsia/nausea	4 (1/3/0)	2 (1/1/0)
	Ulcera - oral	0	1 (1/0/0)
	Ulcera - ileum/colon/rectum	1 (1/0/0)	0
Hepatobiliary/Pancreas	Pancreas irritation[(1)]	0	1 (0/1/0)
Infection	Infection - gastroenteritis	1 (0/1/0)	2 (0/2/0)
	Infection - dental-tooth	1 (0/1/0)	1 (0/1/0)
	Infection - skin	1 (0/1/0)	1 (0/1/0)
Lymphatics	Edema - lower limbs	2 (1/1/0)	0
Metabolic/Laboratory	Elevated AST, GGT	1 (1/0/0)	1 (1/0/0)
	Hypokalemia	1 (0/0/1)	0
Neurology	Dizzyness	1 (1/0/0)	0
	Mood alteration - depression	1 (0/0/1)	0
	Neuropathy - carpal tunnel syndrome	1 (0/1/0)	0
Pain	Abdominal	1 (0/0/1)	0
	Anal/perianal	4 (0/4/0)	1 (0/1/0)
	Joint	0	1 (0/1/0)
	Chest/thorax	0	1 (0/1/0)
Pulmonary/Upper respiratory	Dyspnea	0	2 (0/2/0)
	Nasal cavity/paranasal sinus reaction	0	2 (0/2/0)
Renal/Genitourinary	Lower urinary tract symptoms - prostatism	1 (0/1/0)	0
Secondary malignancy	Secondary malignancy - basalioma - nose	1 (0/0/1)	0
	Total n of reported AE	34 (10/18/6)[(2)]	24 (9/15/0)[(2)]
	Patients reporting ≥1 AE (n, %)	16 (84.2%)	14 (77.8%)

Number of specific adverse event reported during 6 month intervention in patients with FAP with either celecoxib & ursodeoxycholic acid (group A) or celecoxib & placebo (group B), is depicted as grade 1, 2, or 3, as defined by the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. Grade 4 and 5 adverse events did not occur.

[(1)] Adverse event related to pre-intervention duodenoscopy; no other adverse events related to duodenoscopy were reported; [(2)] Distribution of number of adverse events grade 1, 2, or 3, was not significantly different between treatment groups (Fisher's exact, p=0.114). *Abbreviations*: FAP, familial adenomatous polyposis; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; AE, Adverse Event.

DISCUSSION

This randomized controlled trial confirms that celecoxib mono-treatment reduces duodenal polyp density in patients with FAP, whereas it demonstrates that celecoxib and UDCA co-treatment has no beneficial effect. In contrast to our hypothesis of an expected additional effect of the combination, our results imply that the clinical improvement observed in patients treated with celecoxib alone is counteracted by co-treatment with UDCA. We found no changes in cell proliferation, apoptosis or COX-2 expression in normal duodenal mucosa of patients with FAP, that could explain the observed effects.

The clinical improvement of duodenal polyp density after treatment with celecoxib alone, confirms results from a previous trial with similar design.[19] COX-2 overexpression was found in oesophageal [34], gastric [35], colorectal [36], as well as small intestinal cancer.[37] Multiple lines of evidence, including results from *in vitro*, animal, and clinical studies, indicated that inhibition of the increased COX-2 expression, at least in part accounts for the anti-proliferative activity of celecoxib.[38] In addition, COX-2 independent pathways were suggested to be involved in the anti-proliferative effect of celecoxib.[38,39] To our surprise, we found a high COX-2 expression by immunohistochemical analyses, but detected no COX-2 mRNA expression in normal appearing duodenal mucosa of patients with FAP, neither pre- nor post-intervention. Consequently, controversy exists between assessment of COX-2 by immunohistochemistry or qPCR assay. We assume that results on COX-2 in immunohistochemistry could be based on aspecific protein staining by the COX-2 antibody. Assessment of duodenal COX-2 mRNA levels by using Quantigene Plex Assay, confirmed our findings with the qPCR assay: COX-2 mRNA expression is extremely low or even absent in normal duodenal mucosa of patients with FAP (van Heumen *et al.*, unpublished results, **Chapter 7** of this thesis). Our results are in agreement with a previous report of undetectable COX-2 mRNA levels in human small intestinal mucosa by using qPCR analysis.[37]

After prophylactic colectomy, duodenal bile composition changes and largely consists of cholic acid (CA) and chenodeoxycholic acid (CDCA).[40] In *in vitro* models of human colon cancer cells, UDCA significantly reduced cytotoxicity of secondary bile acids.[22] By UDCA supplementation in patients with FAP, up to 50% enrichment of duodenal bile with UDCA was reached, with a large reduction in concentration of the cytotoxic CDCA.[41] Based on these findings, an inhibition of cell proliferation was expected after UDCA supplementation. Although we combined celecoxib and high dose UDCA (20-30mg/kg daily), the *in vitro* effects could not be reproduced *in vivo* in our trial. Moreover, our hypothesis was in part based on clinical studies in patients with UC and PSC showing chemopreventive effects of UDCA on development of colorectal neoplasms.[25,26] Recently however, treatment of patients with UC and PSC with high dose UDCA (28-30mg/kg daily) was found to be associated with an increased risk of colorectal neoplasms.[42] This could be an explanation for the disappointing effect we obtained by the combination treatment of celecoxib and high dose UDCA. In contrast, a recent meta-analysis revealed that long-term low dose UDCA treatment (8-15mg/kg daily) reduces the risk of advanced colorectal neoplasms in patients with UC and PSC.[43] Extrapolating these results, long-term low dose UDCA treatment could be expected to be effective in patients with advanced duodenal adenomatosis. However, in a recent clinical trial in patients with FAP, no effects of low dose UDCA (10mg/kg daily) after 24 months as mono-treatment were found on Spigelman scores.[44] Future studies that focus on the intracellular mechanisms of action may elucidate the ambivalent effect of UDCA as chemopreventive drug in (pre-) clinical studies.

The present study has several strenghts. First, it is the first randomized clinical trial to investigate a combination of two potential chemopreventive drugs for duodenal adenomatosis in patients with FAP. Second, the study population consists of an unique sample of patients with FAP from 5 out of the 8 Dutch University Medical Centres. Third, bias due to interobserver variability was minimized, as primary outcome was based on scores of polyp density by 5 gastroenterologists, who independently compared pre- and post-intervention videos shown in random order, while blinded to treatment allocation. The following limitations are noted. First, our study lacks a 'true placebo' group. Hence, we were not able to confirm the spontaneous reduction in duodenal polyps in the placebo group, that was previously described.[19,44] Second, changes in duodenal polyp density are assessed qualitatively. In previous chemopreventive studies on colorectal adenomatosis, changes in polyp density were assessed by exact counting of polyp number and measuring polyp diameter.[12,45] This method is not suitable for assessment of the plaque-like duodenal polyps, which are partially obscured due to folding over the mucosal folds. Moreover, the curved anatomy of the duodenum introduces an optic bias in the two dimensional images obtained during endoscopy, which further hampers reliable quantification. In clinical practice, the Spigelman scoring system is an established tool to assess duodenal adenomatosis and is commonly used to plan follow up or treatment.[4,46] In clinical science however, the Spigelman score seems insufficiently distinctive to detect subtle changes in polyp density, and it does not account for peri-ampullary adenomatosis specifically.[47] The applied method of assessment in our study, which does include visual assessment of the peri-ampullary region by side-viewing duodenoscopy, permits adequate comparison with previous studies in the field.[12,19,45] Third, although we were able to detect a significant difference in change in duodenal polyp density between the two treatment groups, sample size requirements were not met. As the participants already were under regular endoscopic surveillance and a chemopreventive option to their benefit was the aim of our study, we expected a high willingness to participate in the trial. However, of all eligible patients with FAP under regular surveillance in any of the five participating centres, 31% declined informed consent. Reports of cardiotoxicity of celecoxib[13,14,20] could have withheld patients with FAP to participate. In addition, because of these reports, we applied strict exclusion criteria, leaving out another 24% of patients. We seemingly underestimated the required dedication to participate in the strenuous study protocol, which included a relatively short follow-up interval of 6 months, as compared to regular surveillance intervals of 2-3 years for patients with Spigelman stage II and 1-2 years for patients with Spigelman stage III.[46] Chemopreventive therapies should be well tolerated and have a low toxicity. During intervention period, up to 81% of patients reported at least one adverse event, and 24% of patients discontinued intervention due to adverse events. Altogether, it seems unrealistic to expect that the regimens under investigation in the present study, would be suitable as a life-time chemopreventive treatment.

In conclusion, high dose UDCA co-treatment completely counteracts the positive effect of celecoxib, namely the reduction of duodenal polyp density in patients with FAP. It still needs investigation whether low dose UDCA co-treatment does have a beneficial effect in this respect. The benefit of long term use of celecoxib for duodenal cancer prevention in patients with FAP needs to be weighed against the potential risk of (cardiovascular) adverse events. The search for effective chemopreventive strategies is ongoing and drugs of interest for patients with FAP include sulindac and difluoromethylornithine[48], curcumin and quercetin[49], and eicosapentaenoic acid.[45] Future research has to result in suitable chemopreventive treatment regimes to avoid radical duodenectomy or duodenal cancer.

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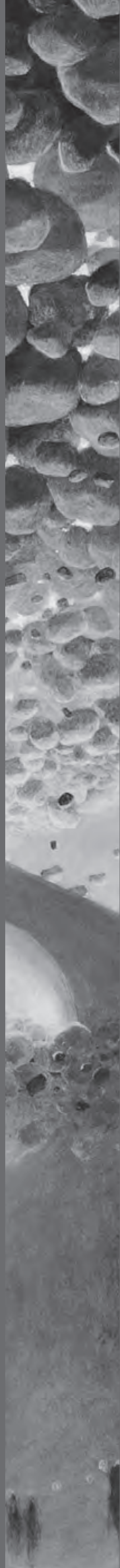

CHAPTER 7

Duodenal mucosal risk markers in patients with FAP: Effects of celecoxib/ursodeoxycholic acid co-treatment and comparison with non-FAP patient controls

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Acknowledgements: The authors thank Dr W Kievit for statistical assistance. Drs Evelien Dekker, Lisbeth Mathus-Vliegen, Jan Dees, Jan Jacob Koornstra and Alexandra Langers are gratefully acknowledged as contributors of the celecoxib/ursodeoxycholic acid randomized clinical trial.

SUBMITTED

ABSTRACT

Chemoprevention would be desirable to avoid duodenectomy in patients with familial adenomatous polyposis (FAP). Identification of risk markers in normal duodenal mucosa could help identify patients at increased risk for malignant transformation. Messenger RNA (mRNA) levels of glutathione S-transferase A1 (GSTA1), glutathione S-transferase P1 (GSTP1), KIAA1199, E-cadherin, peroxisome proliferative activated receptor δ (PPARδ), caspase-3, cyclin D1, β-catenin, and cyclooxygenase-2 (COX-2) were measured using the QuantiGene 2.0 Plex assay. Levels in endoscopically normal appearing mucosa of patients with FAP (n=37) were compared with levels in non-FAP patient controls (n=16). In addition, levels before and after treatment with either celecoxib & ursodeoxycholic acid (UDCA, n=14) or celecoxib & placebo (n=13) were evaluated in patients with FAP. mRNA levels of glutathione S-transferase A1 (28.16% vs. 38.24%, p=0.008) and caspase-3 (3.30% vs. 5.31%, p=0.001) were significantly lower in patients with FAP vs. non-FAP patient controls, respectively. Effect on E-cadherin was significantly different between both treatment groups (p=0.006), however, within each treatment group no statistically significant change in mRNA level was observed. No other statistically significant differences were detected.

In conclusion, protection against toxins and carcinogens (GSTA1) and apoptosis (caspase-3) seems lower in patients with FAP, which could contribute to increased susceptibility for malignant transformation of duodenal mucosa. None of the potential risk markers was consistently influenced by either celecoxib or celecoxib & UDCA. COX-2 mRNA levels in normal duodenal mucosa of patients with FAP, were found to be unexpectedly low.

Keywords: familial adenomatous polyposis (FAP); duodenal mucosa; risk markers; celecoxib; ursodeoxycholic acid; duodenal adenomatosis

Abbreviations: AMC, Academic Medical Centre Amsterdam; *APC,* adenomatous polyposis coli; β2M, beta-2-microglobulin; COX-2, cyclooxygenase-2; EMC, Erasmus Medical Centre Rotterdam; FAP, familial adenomatous polyposis; GSTA1, glutathione S-transferase A1; GSTP1, glutathione S-transferase P1; GSTs, glutathione S-transferases; lef, lymphoid enhancer factor family; LUMC, Leiden University Medical Centre; NSAIDs, non-steroidal anti-inflammatory drugs; PGE2, prostaglandin E2; PPARδ, peroxisome proliferator-activated receptor δ; qPCR, quantitative polymerase chain reaction; RUNMC, Radboud University Nijmegen Medical Centre; Tcf, T cell factor; UDCA, ursodeoxycholic acid; UMCG, University Medical Centre Groningen

Familial adenomatous polyposis (FAP), characterized by the development of numerous premalignant colorectal adenomatous polyps, is caused by a germline mutation in the tumor suppressor *adenomatous polyposis coli* (APC) gene.[1] In the past decades, preventing development of colorectal cancer by prophylactic colectomy, substantially improved prognosis in patients with FAP.[2] As a result, the mortality pattern has changed and duodenal cancer now is the leading cancer-related cause of death.[3, 4] Lifetime risk of duodenal adenomas approaches 100% in patients with FAP[5], and approximately 3-7% of patients eventually develop duodenal cancer.[6, 7] As duodenal cancer in patients with FAP has been associated with a poor prognosis[8, 9], the clinical challenge is to identify patients with high-risk duodenal adenomas and intervene before progression to cancer occurs. Identification of early risk markers in normal duodenal epithelium could help identify patients at increased risk for malignant transformation.

Potentially useful biomarkers can be expected in cellular pathways that are linked to the affected *APC* gene and its translational product. APC is a multifunctional protein involved in regulation of cell proliferation, cell migration, cell adhesion, cytoskeletal reorganisation, and chromosomal stability.[10] The role of APC in intestinal carcinogenesis is attributed largely to the Wnt signaling pathway, but disruption of intercellular adhesion and stability of the cytoskeleton seems to be involved as well.[11] Loss of functional APC results in accumulation of cytosolic β-catenin and subsequent translocation to the nucleus, where β-catenin associates with members of the T cell factor (Tcf) and lymphoid enhancer factor family (lef).[11] The β-catenin/Tcf complex activates several transcriptional targets, including the G1/S-regulating cyclin D1[12] and the peroxisome proliferator-activated receptor δ (PPARδ).[13] In addition, β-catenin also functions as an essential component of epithelial intercellular adherens junctions, where it links the cytoplasmic tail of E-cadherin to α-catenin which binds actin and actin-associated proteins of the microtubule cytoskeleton.[14] KIAA1199 was recently described as a novel target of the Wnt signaling pathway, in both colon and gastric carcinogenesis.[15, 16] While disruptions in the Wnt signaling pathway are involved in tumor initiation[17], abnormal expression of cyclooxygenase-2 (COX-2) observed in the majority of adenomas and carcinomas, is thought to play a crucial role in tumor progression by increasing the levels of prostaglandin E2 (PGE2).[18] Overexpression of COX-2 is linked to reduced apoptosis, enhanced cell growth, tumor angiogenesis, and tissue invasion and metastasis.[19] The involvement of the COX-2/PGE2 pathway may also explain the observed chemopreventive effects of COX inhibiting non-steroidal anti-inflammatory drugs (NSAIDs), decreasing the occurrence of sporadic colorectal adenomas.[20, 21] Treatment with the COX-2 selective inhibitor celecoxib was found associated with regression of colorectal adenomas in patients with FAP.[22] Significant reduction in duodenal polyp density in patients with FAP with clinically significant disease was achieved with high-dose celecoxib[23], a finding we recently confirmed (see **Chapter 6**).[24] Combining celecoxib with other potentially effective drugs could reveal more effective strategies. Based on several preclinical and clinical studies, ursodeoxycholic acid (UDCA) was a candidate drug.[25-29] However, combining celecoxib with UDCA was found ineffective in reducing duodenal polyp density in patients with FAP (see **Chapter 6**).[24]

The clustering of adenomas around the ampulla of Vater suggests that cytotoxic bile plays a role in duodenal adenomatosis in patients with FAP.[30] Detoxification enzymes, such as glutathione S-transferases (GSTs), protect the gastrointestinal mucosa against exogenous and endogenous toxic, mutagenic or carcinogenic compounds by catalyzing the conjugation with glutathione.[31] In patients with FAP, a significantly lower GST activity was observed in colonic mucosa as compared to healthy controls.[32] Distorted expression levels of detoxification enzymes in the duodenum, could reduce functional activity and modulate individual susceptibility for development of duodenal adenomas and carcinomas in patients with FAP.

Aim of the present study was to gain further insight into the cellular targets of potential chemopreventive treatment for duodenal adenomatosis in patients with FAP, as well as to define epithelial risk markers of malignant transformation. We determined messenger RNA (mRNA) expression levels of potential risk markers in duodenal epithelium of patients with FAP in comparison to non-FAP patient controls. Furthermore, we investigated the effects of treatment with celecoxib or celecoxib & UDCA co-treatment on mRNA expression of these selected biomarkers.

PATIENTS AND METHODS

Study participants

The study population consists of patients with FAP and non-FAP patient controls. The patients with FAP, recruited at the Radboud University Nijmegen Medical Centre (RUNMC), Academic Medical Centre Amsterdam (AMC), Erasmus Medical Centre Rotterdam (EMC), University Medical Centre Groningen (UMCG), and Leiden University Medical Centre (LUMC), participated in a double-blind randomized clinical trial (http://ClinicalTrials.gov number NCT00808743), which is described in detail elsewhere (see **Chapter 6**).[24] In short, after completion of pre-intervention gastroduodenoscopy, patients with FAP were randomly assigned to one of two treatment groups. Patients in group A received celecoxib (Celebrex, Pfizer, New York, NY, USA) 400 mg twice daily for 6 months in combination with UDCA (20-30mg/kg body weight daily; Ursofalk, Dr Falk Pharma, Freiburg, Germany). Patients in group B received celecoxib 400 mg twice daily in combination with an UDCA identical-appearing placebo (Dr Falk Pharma). The diagnosis FAP was established either clinically, by the presence of >100 colorectal polyps, or genetically, by the presence of adenomatous polyposis coli (*APC*) gene mutations. Eligible patients were between 18 and 70 years of age, capable of informed consent, had Spigelman stages II or III at last surveillance gastroduodenoscopy, and had no history of surgical duodenal resection.

Patient controls were recruited at the Department of Gastroenterology & Hepatology, RUNMC. All patients aged 18 to 70 years and scheduled for diagnostic gastroduodenoscopy because of dyspepsia, or follow-up after previous diagnosis of upper gastrointestinal dysplasia or Barrett's metaplasia, were selected. With permission from the referring physician, patients received a study information leaflet and an informed consent form by post. They were contacted by telephone one week before the planned gastroduodenoscopy to inquire if additional information was necessary, whether they were willing to participate, and if so, if any of the exclusion criteria were applicable. Exclusion criteria were: use of NSAIDs or UDCA for >1 week during 3 months prior to study entry, history of inflammatory bowel disease, upper gastrointestinal cancer, upper gastrointestinal surgery, celiac disease, pregnancy, or lactation. Informed consent was obtained prior to gastroduodenoscopy. Patient controls were compensated for participation with €100.

From all participants, four random biopsies of normal appearing mucosa were taken in the D2 segment of the duodenum, as well as four random biopsies in the D3/D4 segment. An Olympus Endojaw FB-232U with open forceps diameter 9mm, or a Boston Scientific Radial Jaw 3 with open forceps diameter 8mm (Boston Scientific, Natick, MA, USA) was used. Biopsies were snap frozen in liquid nitrogen and stored at -80°C for mRNA analyses. Patients with FAP underwent gastroduodenoscopy twice: at baseline and after the intervention period.

The present study was conducted according to ICH Good Clinical Practice and complied with the principles of the amended Declaration of Helsinki and Dutch legislation. Ethical approval was obtained at the initiating centre Radboud University Nijmegen Medical Centre (RUNMC; number 2008/148; CCMO number NL23569.091.08). All study participants provided written informed consent. Disclosure of randomization was performed by the Department of Clinical Pharmacy, RUNMC, on December 10th 2012, after all tissue assessments and analyses were completed.

Isolation of RNA from biopsies and quantification of duodenal mRNA levels

One biopsy of each location was weighed and taken up in 200µL TRIzol (Life Technologies, Pailey, UK). Tissue was homogenized by 10 strokes with a Teflon pestle and after homogenization another 600µL TRIzol reagent was added. Total RNA was extracted according to the manufacturer's instructions (Life Technologies) with a slight modification as follows: prior to precipitating the RNA with iosopropyl alcohol, 7.5µg RNAse-free glycogen was added as a carrier to the aqueous phase. Approximately 1µg of total purified RNA was used for the QuantiGene 2.0 Plex assay (Affymetrix, Santa Clara, CA, USA).

RNA was incubated with Luminex beads and capture probes according to the protocol of the manufacturer. Target-specific probe sets for beta-2-microglobulin (β2M, NM_004048), glutathione S-transferase A1 (GSTA1, NM_145740), glutathione S-transferase P1 (GSTP1, NM_000852), KIAA1199 (NM_018689), E-cadherin type 1 (CDH1, NM_004360), peroxisome proliferative activated receptor delta (PPARD, NM_006238), caspase-3 (CASP3, NM_004346), cyclin D1 (CCND1, NM_053056), beta-catenin-1 (CTNNB1, NM_001904) and cyclooxygenase-2 (COX-2, NM_000963) were developed by Affymetrix. Signals of cascade amplification of the fluorescent microspheres were detected by the Biorad Luminex100 Bio-Plex system using the Bio-Plex Manager 4.1 software (Bio-Rad Laboratories, Hercules, CA). The mRNA expression level of the housekeeping gene β2M in each sample was used for normalization. mRNA levels were expressed as percentage relative to the levels of β2M, which were set at 100%. The mean of the mRNA expression levels at the D2 and D3/D4 locations was used in the analyses.

Statistical analysis

Baseline characteristics were expressed as percentage or medians with range where appropriate. Outcome variables were expressed as group medians with 25 and 75 percentiles. Differences on discrete variables were examined using Chi-square test, or Fisher's exact test when appropriate. Continuous variables were considered to be not normally distributed. Differences on continuous variables of baseline characteristics and outcome measurements between groups, including measurements in patients with FAP at baseline versus non-FAP patient controls, and measurements in FAP patients treated with celecoxib & UDCA versus patients treated with celecoxib & placebo, were tested using Mann-Whitney U test. Differences within study groups, comparing pre- and post-intervention measurements in patients with FAP, were examined using Wilcoxon Signed Rank test. For baseline characteristics, a p-value of <0.05 (2-sided) was considered statistically significant. Since each analysis of the outcome measurement comprised a set of nine mRNA expression levels, a correction for multiple testing was applied. In each of these analyses, a p-value of <0.01 was considered statistically significant. Statistical analysis was performed using SPSS statistical software version 21 (IBM SPSS, Chicago, IL, USA).

RESULTS

Patient characteristics

Patient characteristics of patients with FAP and non-FAP patient controls are depicted in ***Table 1***. Thirty-seven patients with FAP were randomized: 19 patients received celecoxib & UDCA (group A) and 18 patients received celecoxib & placebo (group B). Nine patients with FAP (24.3%; group A, n=5; group B, n=4) discontinued intervention prior to post-intervention gastroduodenoscopy at 6 months. In one patient in group B, post-intervention biopsies could

Table 1. Baseline characteristics of the study population consisting of patients with FAP and non-FAP patient controls.

	Overall non-FAP group	Overall FAP group	p-value	FAP group A: Celecoxib & UDCA	FAP group B: Celecoxib & Placebo	p-value
Number of patients	16	37		19	18	
Age at study entry, median/range (yr)	53/23-67	42/22-67	0.092[(1)]	42/22-67	41/27-64	0.964[(1)]
Sex (n, %)			0.241[(2)]			0.618[(2)]
Male	5 (31)	18 (49)		10 (53)	8 (44)	
Female	11 (69)	19 (51)		9 (47)	10 (56)	
Body Mass Index, median/range (kg/m^2)	26.1/ 19.4-44.2	25.6/ 18.8-34.5	0.779[(1)]	26.0/ 19.2-34.5	25.6/ 18.8-33.1	0.408[(1)]
Spigelman score at last surveillance before entry						0.985[(2)]
II		19 (51)[(3)]		10 (53)	9 (50)[(3)]	
III		17 (46)[(3)]		9 (47)	8 (44)[(3)]	

[(1)] The p-value was calculated by the Mann-Whitney U test
[(2)] The p-value was calculated by the Chi-square test
[(3)] In 1 case data on Spigelman score at last surveillance gastroduodenoscopy before study entry was missing
Abbreviations: FAP, familial adenomatous polyposis; UDCA, ursodeoxycholic acid

not be processed and for this patient only pre-intervention measurements were included in the analyses.

Seventeen non-FAP patient controls underwent gastroduodenoscopy as prescribed by the study protocol. One patient control was excluded from analyses, due to diagnosis of celiac disease based on histopathological examination of the biopsies, and 16 non-FAP patient controls were included in the analyses. Indications for gastroduodenoscopy were dyspepsia (n=10), iron deficiency anemia (n=1), follow-up after previous dysplasia or metaplasia in the upper gastrointestinal tract (n=4), and follow-up after Helicobacter pylori eradication (n=1).

Patients with FAP vs. non-FAP patient controls

Median mRNA levels of selected genes in endoscopically normal appearing mucosa of patients with FAP before clinical intervention (n=37) were compared with the levels in non-FAP patient controls (n=16). Results are included in ***Tables 2a*** & ***2b***.

mRNA levels of GSTA1 and caspase-3 were significantly lower in patients with FAP when compared to levels in non-FAP patient controls (GSTA1: 28.16% [25-75%: 21.62%-37.90%] vs. 38.24% [27.25%-51.76%]; Mann-Whitney U, p=0.008); caspase-3: 3.30% [2.46%-4.68%] vs. 5.31% [4.14%-6.77%]; Mann-Whitney U, p=0.001). No statistically significant difference in median duodenal mRNA levels between patients with FAP and non-FAP patient controls were found for GSTP1, KIAA1199, E-cadherin-1, PPARδ, cyclin D1, β-catenin-1, and COX-2.

Patients with FAP pre- vs. post-intervention

Pre- and post-intervention duodenal mRNA levels of selected genes were evaluated in patients with FAP, either treated with celecoxib & UDCA (group A, n=14) or celecoxib & placebo (group B, n=13). Results are shown in ***Tables 2a*** & ***2b***.

Comparison of median pre-intervention mRNA levels of the selected genes indicate that no differences existed at baseline between patients randomly assigned to group A vs. patients randomly assigned to group B (Mann-Whitney U, p>0.05).

The only difference in effect when comparing both intervention groups was found for E-cadherin type 1 (Mann-Whitney U, p=0.006). However, when evaluating the effects within each treatment group, no statistically significant change in mRNA level after treatment was observed, given the correction for multiple testing (group A: median difference = -1.35% [25-75%: -5.00%-0.68%], Wilcoxon Signed Rank, p=0.048; group B: median difference = 1.76% [-0.61%-5.00%], Wilcoxon Signed Rank, p=0.064). For any of the other mRNA levels, the interventions were found to have no statistically significant effects.

DISCUSSION

In the present study, expression of potential risk markers for malignant transformation were assessed by comparing their mRNA levels in normal appearing duodenal mucosa of patients with FAP with levels in non-FAP patient controls. Two important differences were revealed: duodenal mRNA levels of GSTA1 and caspase-3 were significantly lower in patients with FAP as compared to non-FAP patient controls. Lower duodenal levels of the detoxification enzyme

Table 2a. Duodenal mRNA levels of selected genes, expressed as median percentage relative to the mRNA level of the housekeeping gene β2M, set at 100%. Values expressed as mean with 25 and 75 percentiles. (For p-values of statistical analyses comparing mRNA levels of selected genes see Table 2b.)

				Messenger RNA	
			n	**KIAA1199**	**COX-2**
Patient controls			16	0.04 (0.03-0.06)	0.05 (0.04-0.07)
Patients with FAP	Total	Pre-intervention	37	0.05 (0.03-0.24)	0.04 (0.03-0.05)
	Group A: Celecoxib & UDCA	Pre-intervention	19	0.04 (0.02-0.28)	0.04 (0.03-0.06)
		Post-intervention	14	0.04 (0.02-0.23)	0.04 (0.03-0.05)
	Group B: Celecoxib & placebo	Pre-intervention	18	0.09 (0.03-0.22)	0.03 (0.02-0.05)
		Post-intervention	13	0.24 (0.05-0.84)	0.05 (0.03-0.08)

Abbreviations: FAP, familial adenomatous polyposis; UDCA, ursodeoxycholic acid; β2M, beta-2-microglobulin; COX-2, cyclooxygenase-2; CDH1, E-cadherin type 1; PPARD, peroxisome proliferative activated receptor delta; GSTA1, glutathione S-transferase A1; CASP3, caspase 3; CCND1, cyclin D1; CTNNB1, beta-catenin-1; GSTP1, glutathione S-transferase P1.

Table 2b. P-values of statistical analyses comparing duodenal mRNA levels of selected genes. (For duodenal mRNA levels of selected genes see Table 2a.)

		Statistical test
FAP pre-intervention vs. controls		Mann-Whitney U
FAP: pre- vs. post-intervention	Group A: celecoxib & UDCA	Wilcoxon Signed Rank
	Group B: celecoxib & placebo	Wilcoxon Signed Rank
	Group A vs. B: median of differences	Mann-Whitney U

(1) Statistically significant with correction for multiple testing applied ($p<0.01$).
Abbreviations: FAP, familial adenomatous polyposis; UDCA, ursodeoxycholic acid; β2M, beta-2-microglobulin; COX-2, cyclooxygenase-2; CDH1, E-cadherin type 1; PPARD, peroxisome proliferative activated receptor delta; GSTA1, glutathione S-transferase A1; CASP3, caspase 3; CCND1, cyclin D1; CTNNB1, beta-catenin-1; GSTP1, glutathione S-transferase P1.

GSTA1 could point at a lower capacity to detoxify toxins and carcinogens, with subsequent increased susceptibility for malignant degeneration.[31] Previously, we reported a significantly lower GST enzyme activity in colonic mucosa of patients with FAP, as compared to healthy controls[32], but surprisingly, no differences were found in duodenal mucosa of patients with FAP compared to patient controls.[33] However, the sample size in this study was low (n=18), and GSTA1 and GSTA2 were simultaneously measured at the protein level.[33] In contrast, in the present study GSTA1 was selectively measured at mRNA level in 37 patients. GSTP1 levels

Messenger RNA *(continued)*						
CDH1	**PPARD**	**GSTA1**	**CASP3**	**CCND1**	**CTNNB1**	**GSTP1**
16.43 (11.54-22.14)	0.73 (0.52-1.12)	38.24 (27.25-51.76)	5.31 (4.14-6.77)	4.20 (2.71-5.95)	26.90 (21.88-36.03)	9.69 (6.37-15.60)
13.36 (10.82-15.73)	0.71 (0.48-0.95)	28.16 (21.62-37.90)	3.30 (2.46-4.68)	3.72 (3.03-4.69)	24.84 (19.02-28.45)	7.64 (5.70-10.71)
13.54 (10.90-18.51)	0.74 (0.54-1.05)	30.30 (23.16-36.54)	3.30 (2.47-5.21)	3.96 (2.63-4.72)	25.89 (19.80-31.89)	7.59 (5.78-11.07)
13.72 (10.46-15.21)	0.63 (0.50-1.28)	27.13 (21.28-33.30)	3.20 (2.88-4.11)	3.50 (2.81-5.24)	24.05 (20.96-30.27)	7.36 (6.22-10.47)
11.66 (10.28-15.42)	0.63 (0.45-0.87)	27.96 (19.84-39.44)	3.30 (2.42-4.43)	3.63 (3.18-4.63)	21.60 (18.63-27.89)	7.73 (5.46-10.27)
14.08 (10.79-16.87)	0.78 (0.58-1.20)	31.74 (21.02-47.12)	2.94 (2.58-4.83)	4.56 (3.09-5.56)	25.26 (19.93-33.62)	10.82 (7.44-16.18)

P-values								
KIAA1199	**COX-2**	**CDH1**	**PPARD**	**GSTA1**	**CASP3**	**CCND1**	**CTNNB1**	**GSTP1**
0.253	0.021	0.060	0.438	0.008[(1)]	0.001[(1)]	0.373	0.269	0.104
0.730	0.826	0.048	0.778	0.272	0.074	0.730	0.272	0.433
0.046	0.345	0.064	0.279	0.173	0.600	0.075	0.101	0.013
0.048	0.720	0.006[(1)]	0.583	0.048	0.128	0.259	0.043	0.019

were found to be similar in duodenum of patients with FAP and patient controls, which is in accordance with our previous data.[33]

The lower level of caspase-3 found in patients with FAP, can also be considered as risk marker, as it suggests a decrease in apoptosis, with subsequent increased survival of cells with damaged DNA, prone for malignant degeneration. In a recent study, we were unable to detect apoptotic cells by immunohistochemistry in the normal duodenum of patients with FAP (see **Chapter 6**)[24], which is consistent with our current finding using mRNA analysis.

Multiple lines of evidence, including results from *in vitro* studies, animal studies, as well as clinical studies, indicate that inhibition of COX-2 expression, at least in part, accounts for the anti-proliferative activity of celecoxib.[34] By using immunohistochemistry, COX-2 overexpression was reported in oesophageal[35], gastric[36], colorectal[37], and small intestinal cancer[38], as well as in normal duodenal mucosa of patients with FAP.[39,40] Moreover, also by immunohistochemistry, COX-2 levels in normal duodenal mucosa of patients with FAP were reported to be as high as levels in duodenal adenomas or carcinomas, and even higher than levels in normal colonic mucosa.[40] These findings are in sharp contrast with our results from the mRNA analysis. Although we did find high levels of COX-2 mRNA in normal colonic mucosa (Roelofs *et al.*, unpublished results), hardly any mRNA expression was detected in normal duodenal mucosa of either patients with FAP or non-FAP patient controls. Similar results using qPCR analysis to evaluate mRNA expression were previously reported for small intestinal mucosa of non-FAP individuals.[38] These low levels of COX-2 mRNA in duodenal mucosa, in contrast to previous reports using immunohistochemistry, suggest that COX-2 is of minor importance in the initial processes of duodenal tumorigenesis in patients with FAP. Futhermore, the results cast doubt on the specificity of COX-2 detection by immunohistochemical staining.

Loss of functional APC in patients with FAP results in accumulation of cytosolic β-catenin and subsequent translocation to the nucleus[11], where the β-catenin/Tcf complex activates cyclin D1[12] and PPARδ.[13] Our comparison of mRNA levels of cyclin D1 and PPARδ showed similar values in FAP and non-FAP duodenal mucosa ($p=0.37$ and $p=0.44$, respectively). Using immunohistochemistry, expression levels of β-catenin and E-cadherin were found to be lower in normal colon mucosa of patients with FAP as compared to non-FAP controls.[41] In addition, we previously described lower extracellular E-cadherin but higher cytoplasmic β-catenin expression in normal duodenal mucosa of patients with FAP, as compared to non-FAP controls.[42] In the current study, analysis of mRNA levels of β-catenin and E-cadherin markers in normal duodenal mucosa of patients with FAP and non-FAP patient controls could not confirm the previous immunohistochemical findings.

KIAA1199 was reported as a novel target of the Wnt signaling pathway and a putative marker for colorectal and gastic carcinogenic transformation.[15,16] We assessed KIAA1199 mRNA levels to investigate whether this marker is also expressed in the normal duodenal mucosa of patients with FAP with Spigelman grade II or III adenomatosis, consequently being at substantially increased risk of carcinoma development. However, duodenal KIAA1199 levels in normal mucosa of patients with FAP as well as in non-FAP controls were low and comparison did not reveal any difference. This could reflect the physiologic levels of KIAA1199 present at the proliferating crypt basis in the duodenal epithelium, as previously detected in the colonic epithelial crypts.[15] Analysis of KIAA1199 expression in duodenal adenomas could reveal its involvement in duodenal adenomatous transformation.

Recently, we reported that celecoxib & placebo, but not celecoxib & UDCA co-treatment, reduced duodenal polyp density in patients with FAP (see **Chapter 6**)[24]. In that study, cell proliferation, apoptosis and COX-2 expression in the normal duodenal mucosa of the patients with FAP were assessed immunohistochemically, but no effects of celecoxib or celecoxib & UDCA treatment were found. Here, we quantified mRNA levels of several potential risk markers, but again, no consistent effects of either of the two interventions on levels of cell cycle related markers were observed. We did observe a significant difference in change in E-cadherin mRNA levels between both treatment groups, but pre vs. post treatment differences in E-cadherin mRNA levels were not significantly different within either of the two treatment groups. Group comparisons with larger study samples are necessary to elucidate whether actual differences in potential markers do exist.

The present study has several strengths. First, the study population of patients with FAP consists of a relatively large and unique sample from 5 out of 8 Dutch academic medical centers. Second, a new and relatively simple technique, which is not based on quantitative polymerase chain reaction (qPCR), is used to measure mRNA levels of several potential risk markers of interest simultaneously, in normal duodenal mucosa of patients with FAP and non-FAP patient controls. The following limitations are noted. First, the number of non-FAP patient controls included is relatively small. Consequently, group comparisons may lack sufficient statistical power to reveal actual differences, and comparisons in which no statistically significant difference was observed are therefore to be interpreted with caution. Second, mRNA level of each sample was determined as single measurement, however, mean mRNA levels of two different duodenal biopsies from the same patient taken at predefined locations of the duodenum were used in the analyses. Third, although of great interest, we were not able to assess mRNA expression levels in duodenal adenoma biopsy samples. No biopsy samples of adenomas were taken, as pre-intervention sampling of adenomas would have introduced bias in the primary outcome of the intervention study (see **Chapter 6**)[24].

In summary, mRNA levels of nine potential risk parameters for malignant transformation were assessed in normal duodenal mucosa of patients with FAP and non-FAP patient controls. Markers for protection against toxins and carcinogens (GSTA1) and apoptosis (caspase-3) were lower in patients with FAP, which could contribute to the increased susceptibility for malignant transformation of normal duodenal mucosa of patients with FAP. None of the nine evaluated potential risk markers seem to be consistently effected by either celecoxib mono-treatment or celecoxib & UDCA co-treatment. COX-2 levels in normal duodenal mucosa of patients with FAP, measured at the mRNA level, were found to be very low, contrasting previous reports of immunohistochemical findings.

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SECTION D

Discussion, summary in Dutch,
addendum

CHAPTER 8

Summarizing discussion

Abbreviations: AFAP, attenuated FAP; COX, cyclooxygenase; EPA, eicosapentaenoic acid; EMA, European Medicines Agency; FAP, familial adenomatous polyposis; GSH, glutathione; GST, glutathione S-transferase; GSTA1, glutathione S-transferase A1; GSTP1, glutathione S-transferase P1; MAP, *MYH* associated polyposis; NSAIDs, non-steroidal anti-inflammatory drugs; PPARδ, peroxisome proliferative activated receptor δ; UDCA, ursodeoxycholic acid; UGT, UDP-glucuronosyltransferase

Familial adenomatous polyposis (FAP) is an inheritable disease that is characterized classically by the development of hundreds to thousands adenomatous polyps in the colorectum during the second and third decades of life. Virtually all patients with FAP will develop colorectal cancer before the age of 40 to 50 years, unless prophylactic colectomy is performed. In the past decades, performing colectomy as standard prophylactic measure, substantially improved prognosis in patients with FAP. The mortality pattern has changed and duodenal cancer now is one of the main cancer-related causes of death. Practically all patients with FAP develop premalignant duodenal adenomas, which develop to duodenal cancer in approximately 2-7% of patients. Duodenal cancer in patients with FAP has a poor prognosis. The clinical challenge is to identify patients with high-risk duodenal adenomas and intervene before progression to cancer occurs. Prophylactic duodenectomy may offer a prolonged disease-free interval, but is associated with substantial morbidity and mortality. Therefore, chemoprevention would be highly desirable to postpone or even avoid the necessity for radical surgery. In this respect, cyclooxygenase (COX) inhibiting non-steroidal anti-inflammatory drugs (NSAIDs) have been subject of much investigation. Although several studies showed a favorable effect of celecoxib on colorectal adenomas in both patients with sporadic adenomas as well as in patients with FAP, its value in treatment of duodenal polyposis was not well established. The finding that celecoxib significantly reduced duodenal adenomatosis in patients with FAP after 6 months of treatment with high dosage therefore seemed promising. Unfortunately, clinical trials involving selective COX-2 inhibitors as chemopreventive agents for colorectal cancer suggested an increased risk of adverse cardiovascular events. Combining low dose celecoxib with other substances is a suggested alternative strategy in order to minimize toxicity. Several lines of evidence suggest ursodeoxycholic acid (UDCA) to be a candidate for such a combination regimen, but other alternatives are currently explored as well, including curcumin, quercetin, and eicosapentaenoic acid (EPA).

The main objectives of this thesis are:

1. To evaluate the management and its outcome of sporadic duodenal adenomas and duodenal adenomatosis in patients with FAP, as employed in past decades, to further define their clinical significance and implication, and its management.
2. To explore the chemopreventive effects on duodenal adenomatosis of potentially effective substances in a preclinical setting, either as single treatment or in combination, for development of future chemopreventive strategies in patients with FAP.
3. To investigate the chemopreventive effects on duodenal adenomatosis in patients with FAP of treatment with celecoxib and UDCA in a multicentre randomized clinical trial.

The first section of this thesis focuses on the management of duodenal adenomas as it was practiced in the Netherlands in the past decades. The two studies described in this section clarify the clinical significance of duodenal adenomas and illustrate the challenging clinical decisions that patients and their physicians face in the management of duodenal adenomatosis, which is especially evident for patients with FAP. For the study described in **Chapter 2**, the database of the nationwide Dutch polyposis registry in Leiden was used. The centralized registration of patients with FAP in the Dutch polyposis registry not only facilitates patient care by providing a system which supports a strict surveillance program for patients with FAP, but also provides a source of information for retrospective review. For scientific value, completeness of data is essential, and it is therefore of crucial importance that patients and their physicians remain motivated to keep the database complete and up to date. The value of the registered data will further increase by time-lapsing extension of follow-up durations and by reducing bias due to missing data. In **Chapter 2**, the surgical management of severe duodenal adenomatosis in patients with FAP was evaluated, with the primary focus to gain insight into the pros and cons of prophylactic duodenectomy. Patients who underwent (pancreatico-) duodenectomy for advanced benign duodenal adenomatosis, and patients who had presented with duodenal cancer, irrespective of whether duodenal surgery was performed, were identified and included for analyses. A total of 52 patients were classified according to tumor status at preoperative endoscopy, 36 patients with benign duodenal adenomatosis (including two cancer cases diagnosed after operation), and 16 patients with duodenal cancer. Results presented in **Chapter 2** show, that prognosis of duodenal cancer in patients with FAP is poor, justifying an aggressive approach to advanced benign adenomatosis. Although the life time risk of duodenal cancer in Dutch patients with FAP seems modest, morbidity and mortality due to severe duodenal adenomatosis itself as well as resulting from its treatment, is considerable. Our study focused on patients with advanced duodenal disease. However, patients with less severe duodenal adenomatosis are also confronted with a constant cancer threat and periodic endoscopic surveillance, as recommended based on Spigelman staging (see **Chapter 1**, ***Table 1***). Downgrading patient's adenomatosis by radical surgical interventions does not result in exemption from this burden. As adenomas were found to recur in the reconstructed proximal small bowel in 50% of the patients, increased risk of cancer persists and continuous surveillance is warranted. Even more, one patient developed cancer at the hepaticojejunostomy after pancreaticoduodenectomy for duodenal cancer. Altogether, this urges rapid development of strategies to postpone or even prevent necessity of radical surgical interventions, including chemopreventive treatment options, which are subject of the second and third sections of this thesis.

The study described in **Chapter 3** was performed to evaluate management of patients with sporadic duodenal adenomas and to assess the presumed association with colorectal neoplasms. A total of 54 patients, diagnosed with a sporadic duodenal adenoma at our institute between 1986 and 2008, were included in the retrospective evaluation. Although no consistent approach to management of sporadic duodenal adenomas was followed and overall adenoma recurrence after treatment was 20%, no duodenal carcinoma was diagnosed in any of the patients. Endoscopic removal was complete in at least 81% of cases, and no complications were reported. Given the registered complications in surgical intervention in this study, including one complication resulting in death, endoscopic intervention is preferred over surgical intervention, whenever possible. Of note, treatment bias greatly influenced the complication statistics. For patients in whom complete removal is ascertained, no regular follow-up is recommended, especially in elderly patients or patients with relevant co-morbidity. However, an optimal algorithm for treatment and follow-up for all patients with sporadic duodenal adenomas could not be defined. To be able to develop a reliable evidence-based management protocol for patients with sporadic duodenal adenomas, international prospective multicentre studies are necessary, as the incidence of sporadic duodenal adenomas is low. Moreover, duodenal adenomas often are coincidental findings and in most cases their presence is found unrelated to the upper gastrointestinal complaints that were the indication to perform gastroduodenoscopy. Remarkably, a relatively low number of ampullary adenomas were diagnosed, probably due to the fact that endoscopy is generally limited to forward-viewing techniques. However, ampullary adenomas are more likely to undergo malignant transformation compared to adenomas elsewhere in the duodenum. Also given the low estimated prevalence of sporadic duodenal carcinomas in the general population, this implicates that a substantial number of adenomas are never discovered. Therefore, in contrast to duodenal adenomas in patients with FAP, sporadic duodenal adenomas are of less clinical importance.

However, an additional aspect in the management of patients with sporadic duodenal adenomas was underlined by the findings in **Chapter 3**. Colorectal neoplasms, including two cancers and fourteen adenomas, were found in 16 of 29 patients (55%) who also underwent colonoscopy. These findings support the previously reported association between the presence of sporadic duodenal adenomas and colorectal neoplasms. It was suggested that in some patients the association could be based on undiagnosed attenuated FAP (AFAP) or *MYH* associated polyposis (MAP). Nonetheless, a shared common pathway between sporadic duodenal and colorectal neoplasm seems apparent. As FAP proved to be a valuable model in unraveling the genetic processes in the adenoma-carcinoma sequence (see **Chapter 1**, ***Figure 2***) in sporadic colorectal cancer, the same might be true for duodenal carcinogenesis. Based on the findings in **Chapter 3**, colonoscopy is recommended in all patients diagnosed with sporadic duodenal adenomas. Sigmoidoscopy seems a reasonable alternative, as the majority of the associated colorectal lesions were found in the left hemicolon. Future prospective evaluation after implementation of this recommendation in clinical practice is needed to confirm the benefit of colonic assessment in these patients.

In the second section of this thesis, chemopreventive effects of potentially effective substances were explored in human cell line models of intestinal carcinogenesis, in order to develop future chemopreventive treatment strategies for duodenal adenomatosis in patients with FAP. Curcumin, quercetin, and EPA are natural compounds which were found to reduce adenoma burden in patients with FAP. In **Chapter 4**, their effects on the phase II detoxification enzymes UDP-glucuronosyltransferase (UGT), glutathione S-transferase (GST), and glutathione (GSH) were analyzed in four tumor cell line models, in an effort to unravel their mechanism of action. HT-29, HuTu 80, and Caco-2 intestinal cancer cells and LT97 colon adenoma cells derived from a patient with FAP were treated with low-doses of curcumin, quercetin, and EPA. Some enhancing effects on detoxification enzymes of curcumin, quercetin, and EPA were found in Caco-2 and HuTu 80 cells, whereas variable effects were detected in HT-29 and LT97 cells. In conclusion, enhancement of the detoxification enzymes seemed only a minor factor in explaining the anti-carcinogenic properties of curcumin, quercetin, or EPA. However, detoxification enzymes could serve as target for chemopreventive interventions in patients with FAP, as suggested by the results reported in **Chapter 7**, showing that duodenal mRNA levels of glutathione S-transferase A1 (GSTA1) were lower in patients with FAP, as compared to non-FAP patient controls.

In **Chapter 5**, the chemopreventive potential of low dose celecoxib, in combination with UDCA, for (duodenal) adenomatosis in patients with FAP was explored *in vitro*. The effects on cell proliferation, apoptosis and COX-2 expression of both substances were investigated. In this study, LT97 cells derived from a patient with FAP were used, together with the well-established HT-29 human colon adenocarcinoma cell line. Cells were exposed to low dose celecoxib or UDCA alone as well as in combination with taurine conjugated cholic and chenodeoxycholic acid, mimicking bile of patients with FAP treated with UDCA. In HT-29 cells, co-treatment with low dose celecoxib and UDCA resulted in a decreased cell growth (14-17%, $p<0.01$). A more pronounced decrease (23-27%, $p<0.01$) was observed in LT97 cells. Growth of HT-29 cells exposed to 'artificial bile' enriched with UDCA was decreased ($p<0.001$), either in the absence or presence of celecoxib. In LT97 cells incubated with 'artificial bile' enriched with UDCA, cell growth was decreased only in the presence of celecoxib ($p<0.05$). Results from this *in vitro* study show that co-treatment with low dose celecoxib and UDCA has growth inhibitory effects on colorectal adenoma cells derived from a patient with FAP. These findings suggest that celecoxib exerts additional beneficial growth inhibiting effects in FAP-derived adenoma cells and not in carcinoma cells, and further substantiates the bridge between preclinical and clinical results of celecoxib with UDCA as chemopreventive regimen.

Of course, one has to realize that results in cell line models can merely serve as starting point. Relevant effects observed in cell line models have to be further evaluated clinically. The closer the model resembles the *in vivo* setting, the more likely it is that *in vitro* results are relevant *in vivo*. Most human gastrointestinal cell line models originate from colorectal neoplasms, with the human duodenal adenocarcinoma cell line model HuTu 80 as an exception. Unfortunately, our efforts to develop a cell line model derived from a duodenal adenoma of a patient with FAP did not succeed. Such a cell line model could boost investigations of genetic processes in FAP as well as the search for potential chemopreventive strategies. Efforts to establish such a cell line are therefore encouraged.

In the third section of this thesis, clinical chemoprevention is elaborated in two chapters. In **Chapter 6**, the results from a double-blind, randomized clinical trial performed in a collaboration with the Academic Medical Centres of Amsterdam, Rotterdam, Groningen, and Leiden, investigating the effect of celecoxib and UDCA co-treatment on duodenal adenomatosis in patients with FAP, are described. Nineteen patients were treated with celecoxib & UDCA, and eighteen patients with celecoxib & placebo for 6 months. Celecoxib in high doses was once more found to reduce duodenal adenomatosis in patient with FAP. Drug efficacy in the chemoprevention trial was evaluated by independent assessment of pre- and post-intervention duodenal polyp density by blinded review of endoscopic recordings by five gastroenterologists.

Although much effort was put in patient recruitment, even the nationwide collaboration proved insufficient to meet the predefined sample size requirements. In future trials on potential chemopreventive agents, cross-over study designs, in which patients subsequently receive different treatment regimens, could help to increase statistical power without the need for more participants. However, besides order effects and carry-over effects inherent to these study types, an even greater dedication of the participating patient with FAP is required as study duration significantly increases with each additional condition investigated, and subsequent burden increases considerably. Therefore, international collaborations seem the only way to reach study population sizes adequate to evaluate efficacy and safety of chemopreventive treatment for patients with rare diseases.

The number of adverse events in patients treated with celecoxib & placebo as well as celecoxib & UDCA, forcing nine patients (24%) to discontinue intervention prematurely, lead to the conclusion that it seems unrealistic to expect that the chemopreventive regimens studied here will be suitable as life-time chemopreventive treatment. Moreover, in spring 2011, at the same time the last patient completed her intervention period in our trial, the European Medicines Agency (EMA) published the conclusions of their review on the authorization of celecoxib-containing products for use in patients with FAP. In the European Union, celecoxib is authorized for use in osteoarthritis, rheumatoid arthritis and ankylosing spondylitis. The EMA review was initiated after concerns were raised that celecoxib was used off-label after Pfizer voluntary withdrew marketing authorization for use in patients with FAP of its celecoxib-containing orphan medicine Onsenal®. Pfizer decided to do so, as it was unable to provide confirmatory data regarding clinical benefit due to slow enrolment in a clinical trial. Based on available data from published and ongoing efficacy studies and post-marketing safety data, EMA concluded that the benefit of celecoxib in FAP patients had not been sufficiently demonstrated and did not outweigh the increased risk of cardiovascular and gastrointestinal side effects, which could result from high dose and long-term treatment. EMA stated that celecoxib was not to be used off-label in patient with FAP.

Notwithstanding the apparent risk of adverse events, it might still be too soon to discard celecoxib as potential chemopreventive agent, especially since no alternative chemopreventive treatment for this group of patients with increased risk of duodenal cancer is currently available. Celecoxib in low doses combined with other substances might prove to be efficient and safe as chemopreventive strategy. Results from the chemoprevention trial unexpectedly showed that UDCA co-treatment counteracted the favorable effect of celecoxib. Similar to celecoxib, UDCA was also used in high dose, and therefore benificial effects of lower doses in combination treatments are not to be excluded. *In vitro* findings from **Chapter 5** support the assumption of efficacy of low dose UDCA in combination with celecoxib.

In **Chapter 7**, mRNA levels of nine potential risk markers for malignant transformation in duodenal mucosa of patients with FAP are investigated, being: glutathione S-transferase A1 (GSTA1), glutathione S-transferase P1 (GSTP1), KIAA1199, E-cadherin, peroxisome proliferative activated receptor δ (PPARδ), caspase-3, cyclin D1, β-catenin, and COX-2. Levels in endoscopically normal appearing mucosa of patients with FAP were compared with levels in non-FAP patient controls. Levels of GSTA1 and caspase-3 were found to be significantly lower in patients with FAP as compared to non-FAP patient controls. These findings suggest that protection against toxins and carcinogens (GSTA1) and apoptosis (caspase-3) is lower in patients with FAP, which could contribute to increased susceptibility for malignant transformation of duodenal mucosa in these patients.

None of the potential risk parameters was consistently effected by either celecoxib or celecoxib & UDCA co-treatment. COX-2 mRNA levels in normal duodenal mucosa of patients with FAP was found to be unexpectedly low, contrasting previous immunohistochemical reports. This discrepancy in results using different methodology needs further attention, as it might implicate that one or both methods are not valid and conclusions from studies using the invalid method are to be revised. The mechanism of action of celecoxib is complex and encompasses both COX-2 dependent as well as COX-2 independent pathways. In addition, cellular processes targeted by UDCA are also not fully elucidated. Future research is needed to shed more light on these processes to be able to fully understand the observed effects of the substances under investigation here.

CONCLUSIONS

- Prognosis of duodenal cancer in patients with FAP is poor, which justifies an aggressive approach to advanced benign adenomatosis (**Chapter 2**).
- Strict adherence to recommended endoscopic surveillance intervals in patients with FAP is essential for a well-timed duodenectomy to prevent development of duodenal cancer (**Chapter 2**).
- Given the substantial morbidity and mortality of duodenectomy, individual characteristics of patients with FAP are to be critically evaluated preoperatively before such radical surgical intervention is performed (**Chapter 2**).
- As adenomas may recur after duodenectomy, postoperative endoscopic surveillance in patients with FAP is mandatory (**Chapter 2**).
- Even without consistent approach to management, none of the patients diagnosed with sporadic duodenal adenomas developed a duodenal carcinoma during the studied period (**Chapter 3**).
- Whenever possible, endoscopic intervention seems preferable over surgical intervention, surgical interventions could be associated with more morbidity and even mortality (**Chapter 3**).
- Once complete removal of a sporadic duodenal adenoma is ascertained endoscopically, there is no strict indication for regular follow-up, especially in elderly patients or patients with relevant co-morbidity (**Chapter 3**).
- Colonoscopic assessment is warranted in all patients diagnosed with sporadic duodenal adenomas (**Chapter 3**).
- Enhancement of the phase II detoxification enzymes UDP-glucuronosyltransferase and glutathione S-transferase in gastrointestinal adenoma/carcinoma cells by low doses of curcumin, quercetin, or eicosapentaenoic acid seems only a minor factor in explaining the anti-carcinogenic properties of these natural compounds (**Chapter 4**).

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- Co-treatment with low dose celecoxib and ursodeoxycholic acid has *in vitro* growth inhibitory effects on colorectal adenoma cells derived from a patient with FAP (**Chapter 5**).
- Exposure of LT97 and HT-29 cells to ursodeoxycholic acid enriched 'artificial bile', either in absence or presence of celecoxib, suggests that celecoxib exerts beneficial growth inhibiting effects in FAP-derived adenoma cells (LT97) and not in carcinoma (HT-29) cells (**Chapter 5**).
- Celecoxib reduces duodenal polyp density in patients with FAP, and unexpectedly, ursodeoxycholic acid co-treatment counteracts this effect (**Chapter 6**).
- The adverse events that occurred during the 6 months treatment with celecoxib & placebo or celecoxib & ursodeoxycholic acid, require that the benefit of long term use of chemopreventive regimens with celecoxib for duodenal adenomatosis in patients with FAP is weighed against the risk of these adverse events (**Chapter 6**).
- A disturbed protection against toxins and carcinogens caused by low levels of glutathione S-transferase A1 and low apoptotic activity in patients with FAP as compared to non-FAP patient controls, could contribute to the high susceptibility for malignant transformation of duodenal mucosa in patient with FAP (**Chapter 7**).

FINAL CONSIDERATIONS AND FUTURE PERSPECTIVES

Since colectomy has become a standard prophylactic measure in their management, prognosis of patients with FAP has substantially improved. As a result, clinical relevance of duodenal adenomatosis has increased and is expected to further increase with the aging of the current cohort of patients. The clinical significance of duodenal adenomas in patients with FAP, given the considerable morbidity and mortality associated with severe duodenal adenomatosis itself as well as its treatment, require challenging decisions to be made by patients and their physicians. Therefore, the need for chemopreventive drug treatment becomes increasingly urgent. In contrast to high dose celecoxib mono-treatment which reduces the duodenal polyp burden, celecoxib combined with high dose UDCA has no favorable effect on duodenal polyp density. Unfortunately, withdrawal of authorization by the EMA of celecoxib for treatment of adenomatosis in patients with FAP because of the observed adverse events, might hamper further evaluation of the chemopreventive potential of celecoxib. Future investigations should continue to include low dose celecoxib and low dose UDCA, combined with other potential anti-carcinogenic substances, as possible chemopreventive option.

CHAPTER 9

Nederlandse samenvatting

Afkortingen: COX, cyclooxygenase; EPA, eicosapentaeenzuur; FAP, familiaire adenomateuze polyposis; GSH, glutathion; GST, glutathion S-transferase; GSTA1, glutathion S-transferase A1; GSTP1, glutathion S-transferase P1; NSAIDs, non-steroidal anti-inflammatory drugs; PPARδ, peroxisome proliferative activated receptor δ; qPCR, real-time quantitative polymerase chain reaction; UDCA, ursodeoxycholzuur; UGT, UDP-glucuronosyltransferase

HOOFDSTUK 1 (INLEIDING)

Familiaire adenomateuze polyposis (FAP) is een erfelijke ziekte die klassiek gekenmerkt wordt door de ontwikkeling van honderden tot duizenden adenomateuze poliepen in het colon en rectum vanaf de tweede en derde levensdecade. Tenzij profylactisch colectomie wordt uitgevoerd, ontwikkelen vrijwel alle patiënten met FAP vóór de leeftijd van 40 tot 50 jaar darmkanker. Sinds in de voorbije decennia profylactische colectomie als een standaard maatregel wordt uitgevoerd, is de prognose van patiënten met FAP aanzienlijk verbeterd. Naast adenomen in het colon en rectum, ontwikkelen vrijwel alle patiënten met FAP ook premaligne adenomen in het duodenum. Duodenumkanker is momenteel een van de belangrijkste kanker gerelateerde doodsoorzaken. Ongeveer 2-7% van de patiënten ontwikkelt duodenumkanker. De klinische uitdaging in de behandeling van patiënten met FAP is het tijdig identificeren van patiënten met hoog risico adenomen en het ingrijpen voordat progressie van deze adenomen tot kanker is opgetreden, aangezien duodenumkanker een zeer slechte prognose heeft. Profylactische duodenectomie kan een langere ziektevrije periode bieden, maar deze radicale chirurgische ingreep gaat gepaard met een aanzienlijke morbiditeit en mortaliteit. Om de noodzaak voor dergelijke chirurgie uit te stellen of zelfs te voorkomen zou chemopreventie zeer wenselijk zijn. In dit verband is veel onderzoek gedaan naar de effecten van cyclooxygenase (COX) remmers, de zogenaamde “non-steroidal anti-inflammatory drugs” (NSAIDs). In verscheidene studies werden gunstige effecten van celecoxib (een NSAID) aangetoond op adenomen in colon en rectum, zowel in patiënten met sporadische adenomen, als patiënten met FAP. De waarde ervan in de behandeling van duodenale polyposis is echter niet uitvoerig vastgesteld. Dat 6 maanden behandeling met hoge dosis celecoxib bij patiënten met FAP een aanzienlijke vermindering in duodenum adenomen opleverde, leek daarom een veelbelovende bevinding. Helaas lijken klinische studies met selectieve COX-2-remmers als chemopreventie tegen colorectale kanker te wijzen op een verhoogd risico op cardiovasculaire ziekte. Om het risico op deze toxiciteit te minimaliseren zou het combineren van lage doseringen celecoxib met een ander medicament een alternatieve strategie kunnen zijn. Verschillende onderzoeksbevindingen suggereren dat ursodeoxycholzuur (UDCA) een kandidaat is voor een dergelijke combinatiebehandeling. Ook andere alternatieven worden momenteel verkend, waaronder curcumine, quercetine en het omega-3 vetzuur eicosapentaeenzuur (EPA).

De belangrijkste doelstellingen van dit proefschrift zijn:

1. Het evalueren van de behandeling van duodenum adenomen en de uitkomst van behandeling, bij patiënten met sporadische duodenum adenomen en bij patiënten met FAP, zoals dit in de afgelopen decennia in Nederland plaatsvond, om zo de klinische betekenis van duodenale adenomatosis en de behandeling ervan verder te definiëren.
2. Het in preklinische setting verkennen van chemopreventieve effecten van potentieel werkzame middelen, hetzij in enkelvoudig behandeling hetzij in combinatie therapie, om zo toekomstige chemopreventieve behandelingsstrategieën voor duodenale adenomatosis in patiënten met FAP te kunnen ontwikkelen.
3. Het onderzoeken van de theoretisch veelbelovende chemopreventieve behandeling van duodenum adenomen bij patiënten met FAP met celecoxib en UDCA combinatietherapie, middels een multicentrum gerandomiseerde klinische trial.

HOOFDSTUK 2

In het in dit hoofdstuk beschreven onderzoek werd de behandeling en behandeluitkomst voor ernstige duodenum adenomen bij patiënten met FAP retrospectief geëvalueerd. Het primaire doel van de studie was het verkrijgen van inzicht in de voor- en nadelen van profylactische duodenectomie. Bij het nationale polyposis register in Leiden staan meer dan duizend patiënten met FAP geregistreerd. Wij doorzochten de databank van het register en includeerden de volgende patiënten voor onze analyses: (1) patiënten die een radicale (pancreato-) duodenectomie ondergingen in verband met vergevorderde benigne duodenale adenomatosis en (2) patiënten die zich met een duodenumcarcinoom hadden gepresenteerd, ongeacht of duodenale operatie was uitgevoerd. Patiënten werden geclassificeerd volgens tumorstatus bij preoperatieve endoscopie, als patiënten met benigne duodenale adenomatosis of als patiënten met duodenumkanker. Er werden 52 patiënten (25 mannen) geïdentificeerd: 36 met benigne adenomatosis, waaronder twee patiënten die bij operatie gediagnosticeerd werden met kanker, en 16 met kanker. Patiënten die bij preoperatieve endoscopie duodenale kanker hadden, bleken in het verleden vaker gediagnosticeerd te zijn met colorectale kanker dan patiënten die bij preoperatieve endoscopie benigne adenomatosis hadden (44% versus 6%, $p<0{,}01$). In totaal ondergingen 43 patiënten duodenectomie, waarbij mortaliteit en morbiditeit aanzienlijk was. Bovendien ontwikkelden zich bij de helft van de patiënten in de gereconstrueerde proximale dunne darm opnieuw adenomen en in één patiënt zelfs duodenumkanker. De mediane overleving van de 18 patiënten met kanker die in het register werden gevonden was 11 maanden. De resultaten in dit hoofdstuk laten zien, dat de prognose van duodenumkanker bij patiënten met FAP slecht is, hetgeen een agressieve benadering van vergevorderde benigne adenomen rechtvaardigt. Strikte naleving van de aanbevolen intervallen voor endoscopische controle is essentieel voor een goede timing van een eventuele interventie. Gezien de aanzienlijke morbiditeit en mortaliteit van duodenectomie, dienen de individuele kenmerken van patiënten preoperatief kritisch te worden geëvalueerd. Aangezien zich na duodenectomie opnieuw adenomen kunnen ontwikkelen, dient endoscopische controle postoperatief te worden voortgezet.

HOOFDSTUK 3

In dit hoofdstuk worden de resultaten beschreven van het retropectieve onderzoek naar de behandeling van sporadische duodenum adenomen, de uitkomst van de uitgevoerde behandelingen en de aanwezigheid van colorectale tumoren in deze patiënten. Hiertoe werden de medische dossiers van alle patiënten bij wie in ons instituut tussen 1986 en 2008 een sporadisch duodenum adenoom werd vastgesteld retrospectief beoordeeld. Er werden in deze periode 54 patiënten (28 mannen) gediagnosticeerd met een sporadisch duodenum adenoom. Drieëndertig patiënten (61%) ondergingen een endoscopische of chirurgische ingreep, 5 patiënten (9%) werden alleen endoscopisch vervolgd en 16 (30%) ondergingen geen interventie en werden ook niet vervolgd. Volledige endoscopische resectie werd bereikt in 81% van de patiënten en hierbij werden geen complicaties gemeld. Chirurgische interventie verliep gecompliceerd in 4 patiënten (57%), waarbij één patiënt overleed. In 20% van de patiënten werd een recidief adenoom vastgesteld, maar er werd geen carcinoom gevonden. Colorectale tumoren, waaronder 2 kankers (7%), 7 vergevorderde adenomen (24%) en 7 niet-vergevorderde adenomen (24%), werden gevonden in 16 van de 29 patiënten (55%) die ook colonoscopie ondergingen. Hoewel geen consistente aanpak in behandeling van sporadische duodenum adenomen werd gevolgd, ontwikkelde zich tijdens de bestudeerde periode in geen van de patiënten een duodenum carcinoom. Endoscopische interventie lijkt, indien mogelijk, de voorkeur te hebben boven chirurgische interventie, gezien de hierbij gemelde morbiditeit en mortaliteit. Zodra bij de follow-up endoscopie is vastgesteld dat het adenoom volledig is verwijderd, is geen verdere controle meer te adviseren, vooral niet bij oudere patiënten of patiënten met relevante co-morbiditeit. Het is van belang een colonoscopie te verrichten bij alle patiënten die gediagnosticeerd worden met sporadische duodenum adenomen, aangezien duodenum adenomen en colorectale tumoren geassocieerd lijken voor te komen.

HOOFDSTUK 4

In het hier beschreven onderzoek worden de effecten van curcumine, quercetine en EPA op de fase II ontgiftingsenzymen UDP-glucuronosyltransferase (UGT) en glutathion S-transferase (GST)/glutathion (GSH) onderzocht. Om meer inzicht te krijgen in het werkingsmechanisme van deze drie natuurlijke verbindingen in relatie tot hun capaciteit om adenomen bij patiënten met FAP terug te dringen, werden behandeleffecten geanalyseerd in vier cellijn modellen van intestinale kanker, namelijk de darmkankercellen HT-29, HuTu 80 en Caco-2 en de colorectale adenoomcellen LT97, afkomstig van een patiënt met FAP. Een verhogend effect van curcumine en quercetine op GST en UGT expressie werd gezien in Caco-2, LT97 en HuTu 80 cellen. GSH was verlaagd in HT-29 cellen na behandeling met quercetine en EPA en verhoogd in Caco-2 cellen na behandeling met curcumine. In LT97 cellen werd door curcumine en quercetine de GST activiteit en expressie verminderd, de UGT1 expressie verhoogd, terwijl onder invloed van EPA de GST en UGT expressie afnamen. Gezien de ambivalente effecten lijkt een inductie van de ontgiftingscapaciteit geen factor van betekenis om de anti-carcinogene eigenschappen van lage doseringen curcumine, quercetine en EPA te kunnen verklaren.

HOOFDSTUK 5

In dit hoofdstuk wordt de chemopreventieve werking van lage dosis celecoxib in combinatie met UDCA voor (duodenale) adenomatosis bij patiënten met FAP verkend. Doel was het onderzoeken van *in vitro* effecten op de celgroei (celproliferatie en apoptose) en COX-2 expressie van beide stoffen in enkelvoudige behandeling en in combinatie. De humane epitheliale cellijn LT97, verkregen uit colorectale micro-adenomen van een patiënt met FAP, werd als model gebruik. Ter vergelijking werd tevens gekeken in de veelgebruikte humane HT-29 colon adenocarcinoom cellijn. De cellen werden blootgesteld aan lage doseringen van alleen celecoxib of UDCA, combinaties van celecoxib en UDCA, of combinaties van celecoxib en UDCA met 'kunstmatige gal' van patiënten met FAP, bestaande uit taurine geconjugeerd cholzuur en chenodeoxycholzuur. In HT-29 cellen werd een verminderde celgroei (14-17%, p<0,01) gezien na behandeling met lage dosis celecoxib en UDCA. Een meer uitgesproken vermindering (23-27%, p<0,01) werd waargenomen in LT97 cellen. De groei van HT-29 cellen nam af (p<0,001) als de cellen werden blootgesteld aan 'kunstmatige gal' verrijkt met UDCA, al dan niet in aanwezigheid van celecoxib. In LT97 cellen geïncubeerd met 'kunstmatige gal' verrijkt met UDCA, werd alleen een afname in celgroei gezien in aanwezigheid van celecoxib (p<0,05). Resultaten van deze *in vitro* studie tonen aan dat de combinatiebehandeling van lage dosis celecoxib met UDCA groei remmende effecten heeft op cellen van colorectale micro-adenomen van een patiënt met FAP. De bevindingen suggeren bovendien dat celecoxib additioneel gunstige remmende effecten heeft in adenoomcellen van een patiënt met FAP en niet in carcinoomcellen.

HOOFDSTUK 6

In dit hoofdstuk staan de resultaten beschreven van de dubbelblinde, gerandomiseerde klinische trial die werd uitgevoerd in een unieke samenwerking met deskundigen op het gebied van FAP uit de Academisch Medische Centra van Amsterdam, Rotterdam, Groningen en Leiden. In de trial werd de behandeling met celecoxib in combinatie met UDCA vergeleken met celecoxib in combinatie met een placebo, waarbij het primaire doel was om het effect op duodenum adenomen bij patiënten met FAP te bestuderen. Negentien patiënten werden behandeld met celecoxib en UDCA en 18 patiënten met celecoxib en placebo. De behandelduur was 6 maanden. Het effect van de behandeling op de duodenale poliepdichtheid werd beoordeeld door vijf maag-, darm-, leverartsen, door vergelijking van videobeelden vastgelegd tijdens gastroduodenoscopie vóór en ná behandeling. Als secundaire uitkomstmaten werden celproliferatie, apoptose en COX-2 niveaus in normaal duodenum slijmvlies geëvalueerd middels immuunhistochemische kleuringen en/of kwantitatieve polymerase chain reaction (qPCR). Bij intention-to-treat analyse werd een afgenomen poliepdichtheid waargenomen na celecoxib & placebo (p=0,029), terwijl een verhoogde poliepdichtheid werd gezien na celecoxib & UDCA behandeling (p=0,014). De verandering in duodenale poliepdichtheid was statistisch significant verschillend tussen beide groepen (p=0,011). Er werden geen veranderingen in de secundaire uitkomstmaten vastgesteld. Er werd een aanzienlijk aantal bijwerkingen geregistreerd. Negen patiënten (24%) stopten vanwege bijwerkingen zelfs voortijdig met de interventie, 5 patiënten (26%) in de groep die behandeld werd behandeld met celecoxib & UDCA en 4 patiënten (22%) in de groep die werd behandeld met celecoxib & placebo.

De resultaten van de studie tonen aan dat celecoxib de duodenale poliepdichtheid bij patiënten met FAP verminderd, maar dat toevoeging van UDCA aan de behandeling met celecoxib dit effect teniet doet. Bij langdurig gebruik van celecoxib in chemopreventieve behandelschema's voor duodenale adenomatosis bij patienten met FAP dienen de gunstige effecten te worden afgewogen tegen de (risico's op) bijwerkingen.

HOOFDSTUK 7

In de laatste studie in dit proefschrift worden de messenger RNA (mRNA) niveaus van negen potentiële risico markers voor maligne transformatie in duodenum slijmvlies van patiënten met FAP onderzocht. De mRNA-niveaus van glutathion S-transferase A1 (GSTA1), glutathion S-transferase P1 (GSTP1), KIAA1199, E-cadherine, peroxisome proliferative activated receptor δ (PPARδ), caspase-3, cycline D1, β-catenine, en cyclooxygenase-2 (COX-2) werden gemeten met de QuantiGene 2.0 Plex assay, een nieuwe en relatief eenvoudige techniek die niet gebaseerd is op qPCR. Niveaus in endoscopisch normaal-ogend slijmvlies van 37 patiënten met FAP werden vergeleken met de niveaus in 16 niet-FAP controle patiënten. Daarnaast werden de niveaus beoordeeld bij de patiënten met FAP die deelnamen aan de gerandomiseerde studie beschreven in Hoofdstuk 6, vóór en ná behandeling met celecoxib & UDCA (n=14) of celecoxib & placebo (n=13). De mRNA niveaus van GSTA1 en caspase-3 bleken significant lager in patiënten met FAP in vergelijking met niet-FAP controle patiënten. Dit suggereert dat de bescherming tegen giftige en kankerverwekkende stoffen (GSTA1) en het beschermingsmechanisme van geprogrammeerde celdood oftewel apoptose (caspase-3) lager is bij patiënten met FAP. Dit zou kunnen bijdragen aan een verhoogde gevoeligheid voor maligne transformatie van het duodenum slijmvlies bij deze patiënten. De behandeling met celecoxib of celecoxib & UDCA leek geen effect te hebben op de onderzochte potentiële risico markers. De COX-2 mRNA-niveaus in normaal-ogend duodenum slijmvlies van patiënten met FAP was opvallend laag, hetgeen in tegenspraak is met resultaten van eerder onderzoek waarin COX-2-niveaus met immuunhistochemische methoden werden geëvalueerd.

CONCLUSIES

- De prognose van duodenumkanker in patiënten met FAP is slecht, hetgeen een agressieve benadering in de behandeling van vergevorderde benigne duodenum adenomen rechtvaardigt (**Hoofdstuk 2**).
- Strikte naleving van de aanbevolen intervallen voor endoscopische controle in patiënten met FAP is essentieel voor een goede timing van een duodenectomie ter voorkoming van de ontwikkeling van duodenumkanker (**Hoofdstuk 2**).
- Gezien de aanzienlijke morbiditeit en mortaliteit van duodenectomieën in patiënten met FAP, dienen individuele patiëntenkenmerken preoperatief kritisch te worden geëvalueerd voordat een dergelijke ingrijpende chirurgische ingreep wordt uitgevoerd (**Hoofdstuk 2**).
- Aangezien na duodenectomie recidief adenomen in patiënten met FAP frequent voorkomen, dienen ook postoperatief de endoscopische controles te worden voortgezet (**Hoofdstuk 2**).
- Ondanks het feit dat er geen consistente aanpak in de behandeling van sporadische duodenum adenomen is, ontwikkelde geen van deze patiënten tijdens de bestudeerde periode een carcinoom (**Hoofdstuk 3**).
- Waar mogelijk lijkt endoscopische interventie de voorkeur te hebben boven chirurgische interventie, aangezien chirurgische ingrepen worden geassocieerd met meer morbiditeit en zelfs mortaliteit (**Hoofdstuk 3**).

- Zodra volledige resectie van een sporadisch duodenum adenoom endoscopisch is vastgesteld, bestaat er geen strikte indicatie meer voor controle, vooral niet bij oudere patiënten of patiënten met relevante co-morbiditeit (**Hoofdstuk 3**).
- Alle patiënten met een sporadisch duodenum adenoom dienen ook een colonoscopie te ondergaan, gezien de associatie met colorectale neoplasieën (**Hoofdstuk 3**).
- Verhoging van de spiegels van fase II ontgiftingsenzymen UDP-glucuronosyltransferase en glutathion S-transferase in gastrointestinale epitheelcellen door lage doses curcumine, quercetine en eicosapentaeenzuur lijkt geen factor van betekenis in de verklaring van de anti-carcinogene eigenschappen van deze natuurlijke verbindingen (**Hoofdstuk 4**).
- Behandeling met de combinatie van lage dosis celecoxib en ursodeoxycholzuur heeft groei remmende effecten op LT97 colorectale adenoomcellen afkomstig van een patiënt met FAP (**Hoofdstuk 5**).
- Resultaten van blootstelling van colorectale adenoomcellen LT97 en adenocarcinoomcellen HT-29 aan met ursodeoxycholzuur verrijkt 'kunstmatige gal', al dan niet in aanwezigheid van celecoxib, laten zien dat celecoxib additionele groeiremmende effecten heeft in de LT97 adenoomcellen en niet in de HT-29 carcinoomcellen (**Hoofdstuk 5**).
- Celecoxib vermindert de duodenale poliepdichtheid bij patiënten met FAP. Behandeling met celecoxib in combinatie met UDCA doet dit effect echter teniet (**Hoofdstuk 6**).
- Gezien het aanzienlijk aantal bijwerkingen tijdens de 6 maanden behandeling met celecoxib & placebo of celecoxib & ursodeoxycholzuur, is bij een langdurige behandeling met celecoxib voor duodenale adenomatosis bij patiënten met FAP een afweging van de voordelen tegen de risico's op bijwerkingen noodzakelijk (**Hoofdstuk 6**).
- Verminderde bescherming tegen toxische en carcinogene stoffen (lagere niveaus van glutathion S-transferase A1) en verminderde geprogrammeerde celdood (lagere niveaus van caspase-3), zouden kunnen bijdragen aan een verhoogde gevoeligheid voor maligne transformatie van het duodenum slijmvlies in patiënten met FAP (**Hoofdstuk 7**).

AFSLUITENDE OVERWEGINGEN EN TOEKOMSTPERSPECTIEVEN

Sinds colectomie een standaard profylactische maatregel in de behandeling is geworden, is de prognose van patiënten met FAP aanzienlijk verbeterd. Het gevolg is dat de klinische relevantie van duodenale adenomatosis is toegenomen en de verwachting is dat deze met de veroudering van het huidige cohort van patiënten met FAP in de toekomst verder zal toenemen. Dit betekent dat de uitdagende beslissingen waarmee patiënten en hun artsen in de behandeling worden geconfronteerd, door de aanzienlijke morbiditeit en mortaliteit van zowel de ernstige duodenale adenomatosis zelf als de behandeling ervan, alleen maar zullen toenemen. Al met al wordt de behoefte aan medicamenteuze behandeling steeds urgenter. Hoewel de behandeling van celecoxib gecombineerd met ursodeoxycholzuur, beide in hoge doseringen, geen gunstig effect op duodenale poliepdichtheid bleek te hebben, werd het gunstige effect van hoge dosis celecoxib mono-therapie opnieuw vastgesteld. Helaas werd naar aanleiding van de waargenomen bijwerkingen de indicatiestelling van celecoxib voor adenomatosis bij patiënten met FAP ingetrokken. Dit zou een verdere evaluatie van de chemopreventieve potentie van celecoxib kunnen belemmeren. Toekomstige studies dienen lage doseringen celecoxib en UDCA, in combinatie met andere potentiële anti-carcinogene middelen, als mogelijke chemopreventieve opties te blijven evalueren.

ADDENDUM

List of publications
Curriculum vitae
Dankwoord

LIST OF PUBLICATIONS

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CURRICULUM VITAE

BJORN VAN HEUMEN werd geboren op 4 november 1976 te Nijmegen. Hij volgde het Voorbereidend Wetenschappelijk Onderwijs (VWO) aan het Kandinsky College te Nijmegen. Na 3 jaar opleiding geneeskunde aan de Katholieke Universiteit Leuven (België) gevolgd door 2 jaar opleiding psychologie aan de Katholieke Universiteit Nijmegen, werd hij in 2000 toegelaten tot de opleiding geneeskunde aan de Katholieke Universiteit Nijmegen (thans: Radboud Universiteit). Tijdens zijn opleiding geneeskunde kwam hij via het keuzeblok Erfelijke darmkanker in contact met Dr. Nagengast. Onder diens begeleiding maakte hij tijdens zijn wetenschappelijk onderzoeksstage verder kennis met het vakgebied Maag-, Darm- en Leverziekten. Na een afsluitend co-assistentschap in Techiman (Ghana) behaalde hij in 2007 zijn artsexamen. Na zijn afstuderen ging hij aan de slag als arts-assistent-niet-in-opleiding op de afdeling Interne geneeskunde in het Slingeland Ziekenhuis te Doetinchem, waar hij behalve binnen de Interne geneeskunde ervaring opdeed in de Cardiologie, Longziekten en Neurologie. In oktober 2008 maakte hij de overstap naar het Radboud Universiteit Nijmegen Medisch Centrum, in de functie van coördinerend arts-onderzoeker van het KWF-gesubsidieerde onderzoeksproject naar preventie van progressie van duodenale adenomatosis in patiënten met familiaire adenomateuze polyposis (FAP) onder leiding van Dr. Nagengast en Dr. Peters. Op 1 juli 2011 is hij gestart met zijn opleiding tot maag-darm-leverarts (opleider: Prof. Dr. Drenth). In dit kader is hij begonnen met de vooropleiding Interne geneeskunde in het Canisius Wilhelmina Ziekenhuis Nijmegen (opleider: Dr. Dofferhoff). Tijdens deze eerste periode van zijn specialistenopleiding heeft hij zijn promotieonderzoek afgerond, hetgeen heeft geresulteerd in dit proefschrift. Inmiddels heeft hij zijn opleiding voortgezet in het Rijnstate ziekenhuis te Arnhem (opleider: Dr. Wahab). Bjorn woont in Nijmegen samen met Judith van Vliet, neuroloog in opleiding. Zij zijn de ouders van dochter Mirre. In november dit jaar verwachten zij hun tweede kind.

DANKWOORD

De afronding van mijn promotietraject was nooit gelukt zonder de hulp van de volgende mensen.

Dr. Nagengast, beste Fokko. Ik ben je ontzettend dankbaar dat jij mij enthousiast hebt gemaakt voor het vakgebied van de Maag-, darm-, leverziekten en mij de uitvoering van het KWF project hebt toevertrouwd. Regelmatig kwam ik bij je binnen lopen voor overleg. Steevast was je reactie dat je daar nu echt even geen tijd voor had, door drukte rondom patiëntenzorg of onderwijstaken. Maar nadat je mij deelgenoot had gemaakt van je meest recente frustratie, spraken we dan toch altijd even de liggende zaken door. Vervolgens leek je alle werkdruk vergeten en stond je uitgebreid stil bij je ervaringen tijdens een internationaal congres, de laatste aflevering van BBC's Masterchef, of de onovertroffen rijeigenschappen van je nieuwe BMW ('bovendien geweldig dat er ook een studie naar het automerk is vernoemd' [Biomedische Wetenschappen]). Fantastisch. Ontzettend bedankt voor je enthousiasme.

Dr. Peters, beste Wilbert. Ik heb grote bewondering voor je relativeringsvermogen. Wij hebben enorm geworsteld met diverse laboratoriumtechnische problemen. Op alles wat met COX-2 te maken had leek een vloek te rusten. Maar tegenslag dwingt tot heroverwegen en het zoeken naar alternatieve methoden. Hierdoor kan ik mij nu gelukkig prijzen met een laboratoriumervaring die zeker niet voor alle artsen is weggelegd. Als mij de moed zelfs al uit mijn schoenen begon weg te lekken, wist je mij toch te motiveren door te zetten. Immers, elke onderzoeksuitkomst is een rapporteerbaar resultaat. Bedankt voor al je ondersteuning, inclusief tuinonderhoud voor dummy's. En natuurlijk voor jouw (soms onbegrijpelijke) lessen in de wetenschap van het rikken.

Prof. Dr. Drenth, beste Joost. Aangezien mijn promotieonderzoek werd gesuperviseerd door twee zeer ervaren seniorwetenschappers, was jouw rol als promotor inhoudelijk beperkt. Desondanks toonde je veel interesse in de voortgang van mijn onderzoek en gaf je bruikbare adviezen. Veel dank daarvoor. Ik zie uit naar de komende jaren waarin jouw rol als opleider in mijn verdere ontwikkeling tot MDL-arts juist groot gaat zijn. Uiteraard kan ik het niet laten een opmerking te maken over jouw clubvoorkeur. Vooral de clubkleuren stemmen mij uiteraard tot groot ongenoegen. Hoe geweldig is het daarom dat jij, zoals je al lang geleden hebt beloofd, op de dag van mijn promotie gehuld gaat in de kleuren van *mijn* clubvoorkeur. Ik zou haast zeggen dat ik het daarvoor allemaal heb gedaan!

Prof. Dr. Kampman, beste Ellen. Je was betrokken bij de subsidieaanvraag bij het KWF en later bij de opstartfase van mijn onderzoeksperiode en de trial. Je hebt geholpen de zaken op de rails te zetten. Bedankt daarvoor.

Hartelijk dank aan alle patiënten die bereid waren om deel te nemen aan mijn onderzoeken, want zonder deelnemers geen klinische studies en dus geen wetenschappelijke vooruitgang!

De leden van de manuscriptcommissie en overige leden van de corona bedank ik voor hun bereidwilligheid om zitting te nemen in de commissie en zich in mijn werk te verdiepen.

Moeders van mijn promotie. Hennie, ik wens je een verdere carrière zonder COX-2-bepalingen toe! Veel dank voor je oneindige inzet en doorzettingsvermogen. Daar heb ik zeer veel respect voor. Maria, door jouw inspanningen voor de patiëntenzorg van o.a. patiënten

met FAP is wetenschappelijk onderzoek met patiënten met FAP in het UMC St. Radboud haast eenvoudig geworden. Dames, bedankt voor al jullie ondersteuning, en gezelligheid natuurlijk.

Onmisbaar voor het slagen van mijn onderzoeken en het schrijven van de artikelen waren de bijdragen van mijn coauteurs. Vooral de coauteurs van de chemopreventie-trial en hun lokale trialcoördinatoren Christine en Karin (AMC) en Lida, Babs en Maartje (LUMC) zijn er niet makkelijk vanaf gekomen. Zonder jullie tijd en energie waren wij niet tot dit resultaat gekomen. Speciale dank gaat uit naar Iris Nagtegaal, soms een soort derde copromotor, en Elisa, afdeling Pathologie.

Nou ben ik van tijd tot tijd een enorme mopperaar, het is niet anders. Daarom mijn oprechte excuses aan de mensen die mij tijdens mijn fulltime onderzoeksperiode hebben moeten dulden als kamergenoot en mijn al maar intensiverende tirades over eender welke frustratie hebben moeten aanhoren. Eén kamergenoot heeft zelfs overplaatsing aangevraagd... Bedankt Loes, Michelle, Melissa, Ria, Karin en Merel, voor het delen van voorspoed en tegenslag, ik vond het in ieder geval erg geslaagd! Dan wel niet bij mij op de kamer gezeten, maar toch ook altijd in de buurt voor een praatje, een bakkie pleur of een potje rikken, collega's op of rond het lab MDL: René, Jody, Manoe, Evelyn, Wybrich, Wim, en in barrak 'De Buitenhoek': Jannes, Geert, Polat, Mieke, Serena, Martijn, Tom, Robin, Mark, Sanne, dank jullie wel.

Tijdens mijn onderzoek heb ik met studenten geneeskunde en biomedische wetenschappen mogen samenwerken: Marieke, Erik, Maartje, Karlien, Harm, Sanne en Julia. Ik hoop dat het voor jullie net zo leerzaam is geweest als het voor mij was om jullie te begeleiden.

Dames van het secretariaat MDL, dank voor de behulpzaamheid. Hetzelfde geldt voor alle mensen op de endoscopieafdeling (administratief, verpleegkundigen, artsen): er was altijd alle medewerking als ik weer eens aankwam met mijn paparassen, formalinepotjes en container met vloeibare stikstof. Super.

Voor al mijn vrienden ('Leuven', 'Gang 6', 'Geneeskunde Nijmegen', anders): bedankt voor alle gezamenlijke ervaringen en herinneringen, en natuurlijk het ophalen ervan, wat een verrijking! Ik zal er nooit genoeg van krijgen.

Lieve (schoon-)familie, bedankt voor jullie onvoorwaardelijke steun tijdens mijn lange studiecarrière met hindernissen en het begrip dat mijn opleidingssituatie en later werksituatie in combinatie met promotieonderzoek (te) weinig tijd laat voor onze sociale aangelegenheden.

Lieve Judith, hoe kan ik je in een paar regels bedanken voor alle ruimte die je me hebt gegeven naast mijn opleiding mijn promotie tot een einde te brengen. Niet. Ik ben oneindig gelukkig met jou en onze Mirre.